CHANGES IN EXTRACELLULAR MATRIX GENE AND PROTEIN EXPRESSION IN DEVELOPING MOUSE SUBMANDIBULAR GLANDS

BY

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to my wife Beth

for her limitless sacrifices and love

... the very order, changes, and
movements in the universe, the very
beauty of form in all that is visible,
proclaim, however silently, both that
the world was created and also that its
Creator could be none other than God
whose greatness and beauty are both
ineffable and invisible.

St. Augustine, *The City of God*

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CHANGES IN EXTRACELLULAR MATRIX GENE AND PROTEIN EXPRESSION IN DEVELOPING MOUSE SUBMANDIBULAR GLANDS

By

Shawn P. Macauley

May 1996

Chair: Michael G. Humphreys-Beher, Ph.D.
Major Department: Oral Biology

Submandibular gland fetal development involves the orchestrated expression of extracellular matrix molecules (ECM) which direct the morphogenesis, differentiation, and budding of the epithelium. Early morphogenesis of mouse submandibular glands begins on late day 11 of development when the epithelium begins to bud from the surrounding mandibular mesenchyme. Submandibular glands have two bursts of development, one *in utero* and a second postnatally occurring in the suckling/weanling transition. From the small amounts of total RNA collected from fetal BALB/c submandibular glands, gene expression was measured using quantitative competitive RT-PCR. To investigate the changes in ECM gene expression during fetal development, a multiprimer plasmid (pMATRIX) was constructed which contains the primer

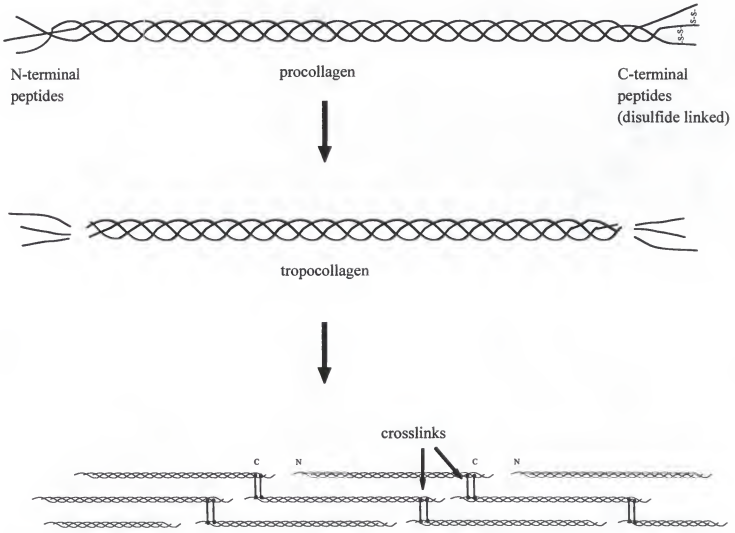
sequences for collagens α2(I), α1(III), α1(IV), fibronectin, laminins B1 and B2, elastin, and lysyl oxidase. The primer sequences were identical for human, rat, and mouse. cRNA was synthesized from the supertemplate and added in varying known amounts to a series of reverse transcription reactions each containing 1.0 μg of total submandibular gland RNA. By comparing the PCR amplification products of both the cellular mRNA and the synthetic template, it was possible to measure the direct expression of each gene. The number of collagen α2(I), collagen α1(III), and lysyl oxidase mRNA molecules per cell peaked on day 16 of development. The expression of elastin mRNA was detectable only on day 16. Fibronectin and laminin B2 were more constitutive in their expression but again had their highest copy number per cell on day 16. A second burst of ECM gene expression occurred in the neonate, with the lowest ECM gene expression seen in the mature adult gland. In addition, immunohistochemistry was performed to localize the protein for each of the ECM molecules examined. With the construction of the pMATRIX supertemplate and the advent of competitive RT-PCR technology it has been possible to measure small changes in ECM gene expression during salivary gland development. This plasmid can be utilized to examine the ECM gene expression of any human, mouse, or rat tissue previously thought to be too small or limited for traditional techniques.

CHAPTER ONE
INTRODUCTION

The Extracellular Matrix

Tissues are not made exclusively of cells but instead derive a substantial part of their volume from the extracellular space which is filled with an intricate network of macromolecules constituting the extracellular matrix (Alberts *et al.*, 1994). Two main classes of extracellular macromolecules make up the matrix: 1) fibrous proteins of two functional types, mainly structural (collagens and elastin) and mainly adhesive (fibronectin and laminin), and 2) glycosaminoglycans (hyaluronic acid, chondroitin sulfate, heparan sulfate and keratan sulfate) which form a highly hydrated gel-like ground substance in which the fibrous proteins are embedded. The macromolecules that constitute the extracellular matrix are mainly produced locally by fibroblasts in the matrix. The assembly, disassembly, and reassembly of extracellular matrices are dynamic processes that occur most rapidly during morphogenesis, growth, and repair (Birk and Linsenmayer, 1994). The extracellular matrix can be divided into at least two distinct components, the interstitial matrices and the basement membranes. Whereas the interstitial stroma possesses thick and long striated collagen cables separated by microfilaments and hydrated glycosaminoglycans, basement membranes are arranged into fine and delicate networks. Basement membranes form thin coats around mesenchymal cells or sheets underneath epithelial and endothelial cell layers called the

Figure 1-1. Structure of fibrillar collagens. Collagen molecules are initially synthesized in the form of pro-α chains which contain extra propeptides on both the carboxy and amino termini. The final collagen molecule contains only the portion of the procollagen molecule in the upper bracket.


N-terminal
peptides
procollagen
C-terminal
peptides
(disulfide linked)
tropocollagen
crosslinks
C
N
C
N
collagen fiber

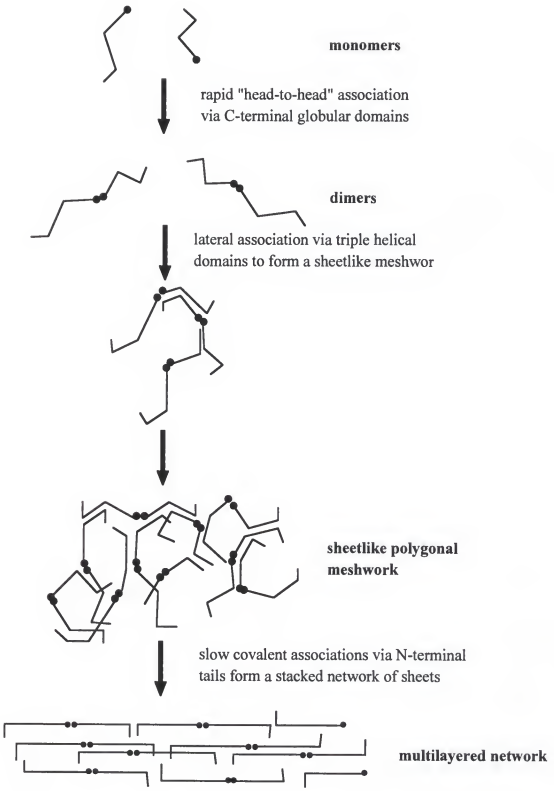
basal lamina (Yurchenco, 1994).

Basement membranes play several roles: they supply mechanical support for cell layers, form flexible barriers between tissue compartments that helps regulate the exchange of macromolecules, serve as interactive surfaces providing adhesion, regulate cell shape, and communicate information for the regeneration and differentiation of cells (Yurchenco, 1994). Discussed briefly below are several major extracellular matrix molecules that are critical for development.

Collagens types I and III. Type I collagen is the most abundant collagen accounting for 90% of all the collagens. Collagens in general account for 25% of the total protein in mammals. Collagens are extremely rich in proline and glycine, both of which are important in the formation of the triple-stranded helices. The general amino acid sequence of collagens is a repeat of $(Gly\text{-}X\text{-}Y)_n$, where X and Y can be any amino acid although one is commonly proline. The high glycine content allows collagen to tightly pack in the triple-helix while prolines stabilize the left-hand helical structure. Fibrillar collagens are secreted into the extracellular matrix by fibroblasts where they form rigid, triple-stranded helical structures made up of three alpha chains. These triple-helical collagen molecules assemble into fibrils which can further aggregate into collagen fibers (Figure 1-1).

Collagen IV. The major molecular constituent of basement membranes is collagen IV along with laminin, entactin and proteoglycans. As is shown in Figure 1-2, each monomer is composed of two $\alpha 1$ chains and one $\alpha 2$ chain. Unlike the fibrillar collagens the regular Gly-X-Y repeating amino acid sequence of type IV collagen is

Figure 1-2. Structure of type IV collagen. Type IV collagen assembles into a multilayered network, which forms the core of all basement membranes.


monomers
rapid "head-to-head" association
via C-terminal globular domains
dimers
lateral association via triple helical
domains to form a sheetlike meshwor
sheetlike polygonal
meshwork
slow covalent associations via N-terminal
tails form a stacked network of sheets
multilayered network

interrupted in a number of regions and each triple-helical chain is longer and more flexible (Hofmann *et al.*, 1984). Rather than assembling into fibrils, type IV collagen usually forms a sheetlike meshwork by forming head to head dimers that is found exclusively in the basal laminae. Type IV collagen dimers appear to assemble initially into a network through noncovalent interactions and quickly becomes crosslinked through disulfides and lysyl oxidase-derived covalent bonds.

Fibronectin. Fibronectin-containing extracellular matrices play critical roles in such diverse processes as embryogenesis, wound healing, and malignant transformation (Peters and Mosher, 1994). Unlike most other commonly studied extracellular matrix proteins, fibronectin does not readily self-assemble and is thought to involve interactions among fibronectin, cell-surface receptors, and other matrix proteins (Peters and Mosher, 1994). Fibronectin is a 500,000 Da multifunctional glycoprotein which can be found as a soluble protein in blood, cerebrospinal fluid, and amniotic fluid or as an insoluble molecule incorporated into the connective tissue stroma, basement membrane, bone, cartilage, and plasma (Hynes, 1985). Fibronectin, shown in Figure 1-3, is made from two similar 250,000 Da subunits which are held together by two disulfide bonds at their carboxy termini (Kar *et al.*, 1993). Numerous isoforms of fibronectin are made as a result of alternate splicing of the fibronectin precursor mRNA and can exist as a homodimer of two identical subunits or as a heterodimer of two similar but unidentical subunits (Petersen *et al.*, 1989). The subunits are flexible and can be found fully extended to form a V-shaped molecule (Emch *et al.*, 1992) or folded into a compact globular molecule (Erickson and Carrell, 1983). Fibronectin can bind to most collagens as well as a number

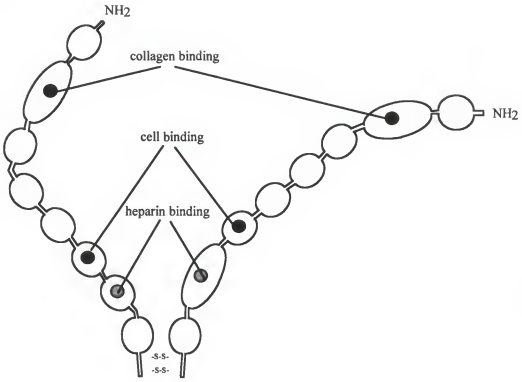


Figure 1-3. Structure of fibronectin. A fibronectin dimer is made of two similar polypeptide chains and are joined by two disulfide bonds near the carboxy terminus. Each chain is folded into a series of globular domains connected by flexible polypeptide segments. Individual domains responsible for binding collagen, heparin, and the cell are shown.

of other ECM components including heparin/heparan sulfate, bone, and fibrin and promotes the attachment of fibroblasts to the matrix in connective tissue (Engvall and Rouslahti, 1977; Engvall *et al.*, 1978, 1982; Jilek and Hörnmann 1979; Woodley *et al.*, 1983; Saunders and Bernfield, 1988; Yamada *et al.*, 1992) and has been shown to co-localize with collagens type I, II, III, laminin, and heparan sulfate proteoglycans in culture (Furcht *et al.*, 1980; Hayman *et al.*, 1981; Hayman *et al.*, 1982; Hedman *et al.*, 1982; Ledger *et al.*, 1980; Little and Chen, 1982; McDonald *et al.*, 1982; Singer *et al.*, 1987; Vaheri *et al.*, 1978). Fibronectin has been implicated in cell migration and morphology, cytoskeletal organization, and control of cell growth and differentiation. Fibronectin is found in various fetal tissues, including connective tissues and basement membrane (Thiery *et al.*, 1989). They are mostly absent from adult tissues but are re-expressed during wound healing and in tumor stroma (Carnemolla *et al.*, 1989; Colvin, 1989; Matsuura and Hakomori, 1985; Vartio *et al.*, 1987).

Laminin. Laminin is the most common non-collagenous protein in the basement membrane and has several *in vivo* functions which include promoting attachment of epithelial cells to the basement membrane, aiding in cell migration during development, and regulating cell growth and differentiation (Drago *et al.*, 1991; Panayotou *et al.*, 1989; Reh *et al.*, 1987; Sweeney *et al.*, 1990). As is shown in Figure 1-4, laminin is an 800 kDa multidomain glycoprotein composed of three genetically distinct polypeptide chains (A, B1, and B2 chains) connected to each other in an asymmetrical four-armed structure by disulfide bonds (Beck *et al.*, 1990; Timpl, 1989). The laminin amino acid sequence shares homologous repeats with EGF (Sasaki and Yamada, 1987). Laminin is

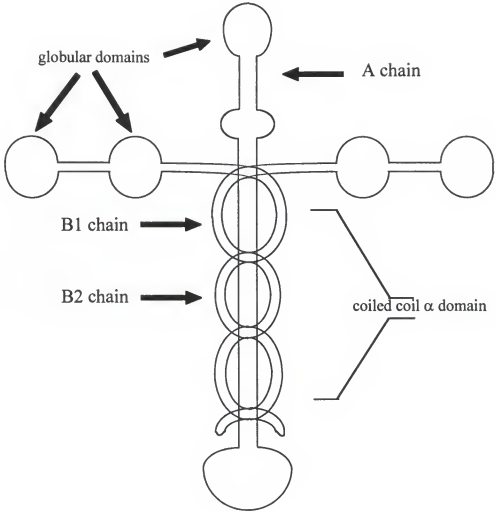


Figure 1-4. Structure of laminin. The muiltidomain glycoprotein is composed of three polypeptides (A, B1, and B2) arranged in an assymetric cross-like structure by disulfide bonds. Each of the polypeptide's chains is more than 1500 amino acids in length.

synthesized by various types of cells including epithelial and endothelial cells, muscle cells, and fibroblasts (Campbell and Terranova, 1988; Paulsson, 1992). Laminin is among the earliest detectable ECM proteins during development appearing at the two-cell embryo stage (Cooper and MacQueen, 1983; Dziadek and Timpl, 1985; Leivo *et al.*, 1980). Messenger RNA expression studies have shown that various laminin chains are temporarily expressed during development and exhibit cell- and tissue-specific patterns of expression (Ekblom *et al.*, 1990; Klein *et al.*, 1990). Laminin binds to type IV collagen, heparan sulfate proteoglycan and heparin (Paulsson, 1992). Laminin interacts with the members of the integrin class of transmembrane receptors (Paulsson, 1992) which are known to mediate the signal to the cytoskeleton influencing cellular behavior, *e.g.*, proliferation and differentiation. It has also been suggested that laminin plays a part in integrating signaling systems with other regulatory molecules such as growth factors (Nathan and Sporn, 1991).

Elastin. Elastic fibers are among the most complex structures of the extracellular matrix requiring at least four different proteins that not only direct polymerization of the elastin monomer but also determine the final architecture of the fiber (Mecham and Davis, 1994). Elastin is the predominant protein of mature elastic fibers and endows the fiber with the characteristic property of elastic recoil. As is shown in Figure 1-5, elastin is secreted from the cell as a soluble precursor protein of 60,000-70,000 molecular weight. Like collagen, a critical step in the maturation of elastin in the covalent crosslinking by lysyl oxidase.

Lysyl oxidase. The deposition of collagen fibrils into specific spatial patterns represents a central problem in morphogenesis. Elucidating the mechanisms regulating

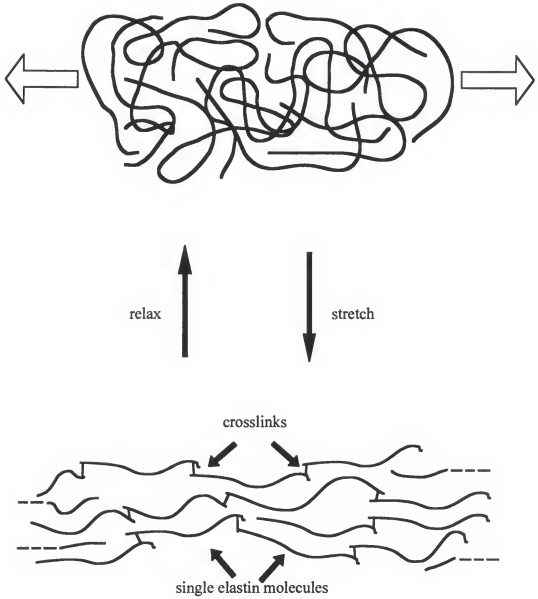
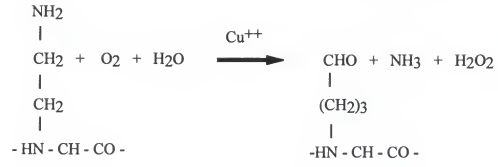


Figure 1-5. Structure of elastin. Elastin moleculaes are held together by covalent bonds to generate an extensive cross-linked network. Some of the covalent bonds are generated by lysyl oxidase.



peptidyl lysine

α-aminoadipic-δ-semialdehyde
"allysine"

Figure 1-6. Crosslinking reaction catalyzed by lysyl oxidase. Crosslink formation is initiated by the oxidative deamination of the ε-amino groups of specific lysyl and hydroxylysyl residues. This forms reactive allysyl and hydroxyallysyl intermediates, which undergo a series of spontaneous nonenzymatic condensation reactions to form novel covalent crosslinks in both collagens and elastin.

collagen fibril assembly, deposition, and growth is necessary to understand the maintenance of structure and tissue function during development. Lysyl oxidase (LO) initiates the enzymatic crosslinking of both collagen and elastin in the extracellular matrix (Kagan, 1986). LO is a copper- and pyrolloquinoline quinone (PQQ)-dependent enzyme which catalyzes the oxidative deamination of ε-amino groups in some lysyl and hydroxylysyl residues of collagen and in lysyl residues of elastin (Figure 1-6) (Siegel, 1979). LO is specific for lysyl residues in collagen and elastin and does not readily deaminate free lysine (Siegel *et al.*, 1970). The deamination of lysyl residues results in the formation of reactive aldehydes (allysine or hydroxyallysine if the substrate residues are lysine or hydroxylysine, respectively) that non-enzymatically and spontaneously condense with lysyl or hydroxylysine residues in adjacent collagen and elastin molecules to form intra- and inter-chain divalent crosslinks. Soluble pro-collagen and pro-elastin secreted into the extracellular matrix is rendered insoluble through the cross-linking activity of LO. The crosslinkages of collagen and elastin contribute significantly to the elastic properties and tensile strength of lung, vascular, and connective tissues. The ratio of collagen to lysyl oxidase and different patterns of temporal expression are thought to be important in influencing the character of the matrix (Birk and Linsenmayer, 1994).

Glycosaminoglycans. Glycosaminoglycans (GAGS) are long, unbranched polysaccharide chains composed of repeating disaccharide units with one unit always being either *N*-acetylglucosamine or *N*-acetylgalactosamine covalently linked to a core protein (Figure 1-7). GAGS are highly negatively charged attracting cations which make them highly hydrophilic, thus occupying very large volumes compared to their mass. The

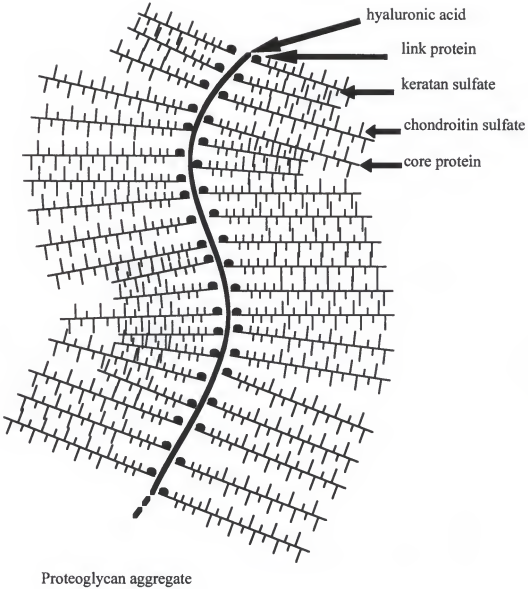


Figure 1-7. Structure of glycosaminoglycans. Schematic drawing of a proteoglycan aggregate consists of approximately 100 proteoglycan monomers noncovalently bound to a single hyaluronic chain. The proteoglycans are attached to the hyaluronic acid chain by link proteins.

amount of GAGS in connective tissue is usually less than 10% by weight of the fibrous proteins, but because they form porous hydrated gels, GAG chains fill most of the extracellular space thus providing mechanical support to tissues while still allowing the rapid diffusion of water-soluble molecules and the migration of cells.

Salivary Gland Development

Salivary glands provide a model system to study many biological phenomena including structure and regulation of living systems. Fetal salivary glands have been utilized for years as a model for mammalian development (Nakanishi *et al.*, 1986). The major salivary glands, *i.e.,* the parotid, submandibular, and sublingual glands, are large paired organs that lie outside the oral cavity proper, and which communicate with the oral mucosa through major ducts. Salivary glands are an ideal system to study development because they are anatomically distinct and as they develop, begin to synthesize and secrete unique salivary-specific proteins. In the mouse, the earliest evidence of submandibular gland morphogenesis begins on late day 11 of development with a focal clustering of the primitive oral epithelium which pushes into the surrounding mesenchyme. Simultaneously, the adjacent mesenchymal cells become more numerous and condense. The epithelium proliferates and begins to bud from the surrounding mandibular mesenchyme on day 12. This occurs when clefts form in the distal end of the terminal bulb which deepen until two or more clefts have developed from the original. This forms a club-shaped structure surrounded by a basement membrane and a thin layer

of oriented mesenchyme. The ducts and alveolar components of the glands are epithelial outgrowths while the stroma and blood vessels are of mesenchymal origin. On day 13 the glands begin to resemble the familiar stalk and branch morphology and can be removed by microdissection. The minor salivary glands begin development as proliferations of oral epithelium in a fashion similar to the major salivary glands.

The most extensive early studies on the embryonic development of salivary glands were carried out in the pig (Chievitz, 1885; Flint, 1903; Kallius, 1910; Moral, 1913a; Moral, 1913b), in the mouse (Moral, 1916; Moral, 1919) and more recently and more precisely in the rat (Cutler and Chaudhry, 1973a; Cutler and Chaudhry, 1973b; Cutler and Chaudhry, 1974; Cutler and Chaudhry, 1975; Redman and Sreebny, 1970a; Redman and Sreebny, 1970b). The literature on salivary gland development has been difficult to compare since Redman and Sreebny (Redman and Sreebny, 1970a) refer to the day after matings as "day zero" whereas Cutler and Chaudhry (Cutler and Chaudhry, 1973a) define the day after mating as "the first day" so that apparent discrepancies between the two studies arise (Young and Lennep, 1978). In describing developmental timing of mouse salivary glands in this discussion the designation made by Cutler and Chaudhry will be used such that the day after the mating is designated as day 1.

There are four key events in the development of salivary glands: 1) morphogenesis, the initiation of the characteristic clefts and branching architecture of the glands leading to lumenization of the primitive glandular epithelium, 2) cytodifferentiation, the differentiation of the rudimentary salivary gland into several cell types which begins after the initial phases of morphogenesis and includes the appearance

and accumulation of secretory granules and other organelles associated with the synthesis, storage, and secretion of specialized salivary-specific protein within the branching structure, 3) development of the stimulus/secretion coupling system by the parasympathetic and sympathetic branches of the autonomic nervous system and the acquisition by the glandular epithelia of receptors to the neurotransmitters of these nerves which together transduce external stimuli into a secretory response by the secretory cells, and 4) the anatomical coupling of the sympathetic nerves to begin the secretion of salivary fluids and proteins (Redman, 1987). In this report the effects of the extracellular matrix on the first two events, morphogenesis and cytodifferentiation, will be discussed.

Salivary Gland Morphogenesis

Role of Epithelial-Mesenchymal Interactions in Salivary Gland Morphogenesis

Classical studies of experimental embryology demonstrated that vertebrate organ development depends on sequential and reciprocal interactions between tissue layers (Grobstein, 1953c; Saxén *et al.*, 1976; Speman and Mangold, 1924). These inductive interactions (or secondary inductions) occur between epithelial and mesenchymal tissues and they lead to differences between cells and to the organization of cells into tissues and organs (Vainio *et al.*, 1993).

There is limited information available concerning the molecules involved in the induction and regulation of the initial events in salivary gland development (Cutler, 1993). Figure 1-8 shows the early *in vitro* development of mouse submandibular glands. The important role of the mesenchymal capsule in the regulation of the development of

the epithelial part of the submandibular gland was first noted by Borghese (Borghese, 1950) when he observed that branching morphogenesis and growth of the submandibular gland rudiment *in vitro* was inhibited if the surrounding mesenchymal capsule (Figure 1-9) was removed. This observation was confirmed and expanded by Grobstein (1953a, 1953b, 1953c) who showed that the potential for branching morphogenesis resided predominantly in the end-buds while the stalk of duct had only limited capacity for branching. In addition, in now classical *in vitro*, transfilter experiments, Grobstein (1953c) demonstrated that the induction of branching morphogenesis appeared to be dependent upon a diffusible macromolecule which was produced by the capsular mesenchyme. Experiments in which the mesenchymal and epithelial elements of salivary gland rudiments were separated by gentle enzymatic digestion, then grown *in vitro* in various combinations from a different gland, established that, in general, salivary glands are highly selective with regard to the sources of mesenchyme that will support their growth and differentiation (Ball, 1974; Lawson, 1974). Bernfield and coworkers (1984) examined the reciprocal interactions between the mouse embryo submandibular epithelium and mesenchyme and found that the interactions modify the composition and metabolism of the basement membrane. They demonstrated that the mesenchyme remodels the basement membrane (BM) by depositing a collagen type I-rich matrix on the basal lamina and by producing a hyaluronidase which degrades hyaluronate and chondroitin sulfate. Similarly BM remodeling may be a general process for regulating cell behavior during other aspects of development. To clarify whether the elongation and branching morphogenesis of salivary glands is directed by the epithelium of the

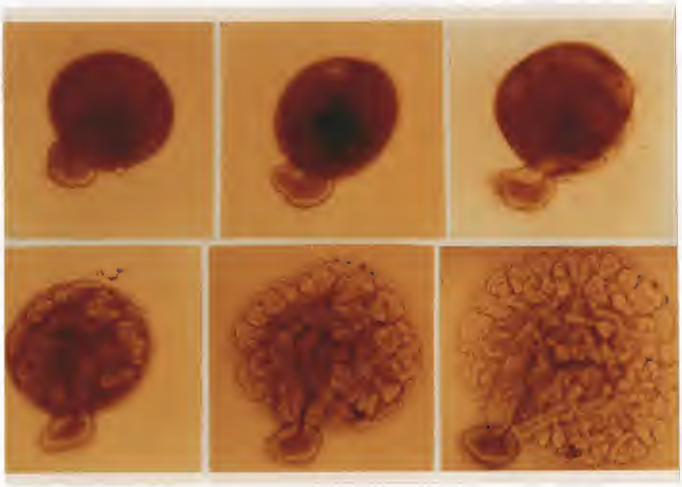


Figure 1-8. *In vitro* development of a fetal mouse submandibular gland. A developing salivary gland was removed at day 13 from a BALB/c fetus. The gland was cultured in DMEM with 10% FCS. The each panel represents the same gland at 0 (upper left), 12 (upper middle), 24 (upper right), 36 (lower left), 48 (lower middle), and 60 (lower right) hours.

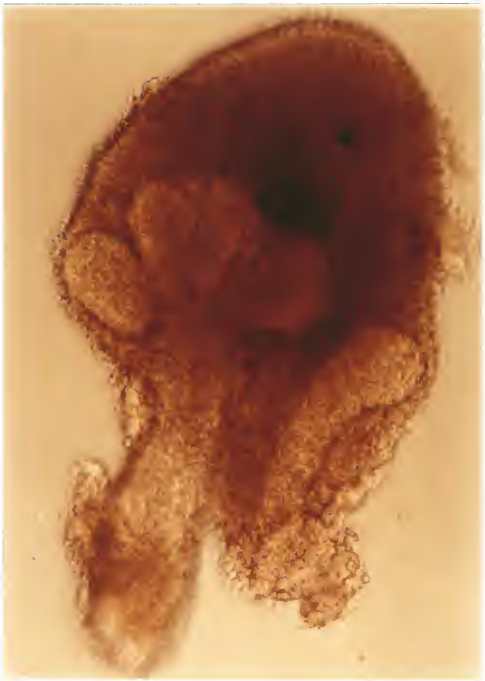


Figure 1-9. Photograph of a freshly dissected day 13 fetal mouse submandibular gland. The submandibular gland epithelium is embedded by the darker appearing mesenchymal capsule.

mesenchyme, Nogawa and Mizuno (1981) demonstrated by recombinational experiments that the elongating and branching morphogenesis of quail salivary glands is controlled by the mesenchyme.

It has been clearly demonstrated that the mesenchymal capsule surrounding the epithelial portion of the salivary gland directly controls the development of the branching pattern seen in the developing glands (Lawson, 1970, 1972, 1974; Grobstein, 1953a, 1953b, 1953c; Bernfield *et al.*, 1972; Cohn *et al.*, 1977). The mesenchyme appears to exercise this control through the selective production and destruction of specific extracellular matrix molecules [Bernfield *et al.*, 1984; Thompson and Spooner, 1982, 1983; Spooner *et al.*, 1985; Grobstein, 1953a, 1953b, 1953c). These extracellular matrix molecules are deposited in and near the basal lamina which serves as the interface between the epithelial cells and their investing mesenchymal capsule.

Borghese (1950) and Grobstein (1953b) showed that mechanical or enzymatic removal of the surrounding submandibular mesenchyme reduced or eliminated the subsequent epithelial branching in culture. However, recombination of the separated mesenchyme and epithelium brought about further proper morphogenesis. This epithelial-mesenchymal interaction is thought to be achieved through the action of extracellular matrix components including basal lamina located between the epithelium and mesenchyme. These components include collagen, proteoglycan, fibronectin, and laminin (Trelstad, 1984). However one of the difficulties in elucidating the precise physiological function of these molecules is that most, if not all, of them exist as a sturdy multi-molecular complex in a solid state. Although the importance of epithelial-

mesenchymal interactions for the development of most organs is well established, the actual mechanisms involved in mediation of inductive signals are only starting to be elucidated.

Contribution of the Extracellular Matrix in Salivary Gland Morphogenesis

The mouse embryonic submandibular gland is an interesting and useful model, for several reasons, for analyzing the mechanisms of epithelial branching. The rudiment can be cultured on a Millipore filter or agar and the change in epithelial shape is easily followed in the living state with light microscopy. Secondly the epithelium and mesenchyme are readily separated from each other by brief treatment with collagenase or other proteases, a property that makes various recombinational experiments possible. Thirdly, because of the absence of the mesothelial layer outside the mesenchyme, proteinaceous factors added in culture medium (*e.g.,* extracellular matrix molecule-degrading enzymes) quickly diffuse into the tissues (Nakanishi and Ishii, 1989).

The ECM can be functionally divided into the basement membrane, a continuous layer underlying all epithelia, and interstitial matrix which forms the noncellular part of the mesenchymal stroma. Each compartment has a distinct set of extracellular matrix macromolecules. For example, basement membranes contain collagen type IV, heparan sulfate proteoglycan, and laminin; while interstitial matrix generally contains mixtures of collagen types I and III, chondroitin sulfate proteoglycan and fibronectin.

The role of the extracellular matrix in the embryological development of the mouse salivary glands has been initially explored. As early as 1965 it was thought that

collagen inhibited or retarded cellular growth -- serving either as an anchor or as a stabilization point around which nonstabilized cells would proliferate, giving rise to new branches (Grobstein and Cohen, 1965).

Collagen appeared to serve as a stabilizing component which inhibited cell growth in specific areas of the growing rudiment while other areas were devoid of collagen and thus able to grow. The stabilized areas could therefore serve as branch points from which morphogenetically active cells gave rise to new branches. These works of Grobstein and Cohen suggested that collagen could reduce cell growth at the branch point and serve as an anchor point from which cells not inhibited by collagen could undergo mitosis and develop into new branches. It was also postulated that some type of localized collagenolytic activity might be involved at the points of growth to free some cells from the stabilizing effect of collagen.

Collagen is present in the mesenchyme and on the epithelial surface, especially on the cleft and stalk regions, and is an important molecule in the branching morphogenesis of the salivary gland (Spooner and Faubion, 1980). To show that collagen was important in epithelial branching, Spooner and Faubion (1980) used L-azetidine 2-carboxylic acid and α,α'-dipyridyl, inhibitors for collagen synthesis and secretion, to introduce collagenolytic activity in the salivary gland. Previous work using bacterial collagenase preparations (Grobstein and Cohen, 1965; Wessels and Cohen, 1968) were possibly obscured due to the probable contamination of mucopolysaccharidases (Bernfield and Banerjee, 1982). Nakanishi and coworkers (1986) also showed that collagen was an important extracellular matrix protein in the regulation of cleft formation in the salivary

epithelium through the use of a collagenase. In that study collagenase from *Clostridium histolyticum* was shown to inhibit cleft formation while a collagenase inhibitor isolated from the culture medium of bovine dental pulp (Kishi and Hayakawa, 1984), now thought to be a tissue inhibitor of metalloproteinases (TIMP), stimulated multiple cleft formations by specifically rearranging type III collagen to allow cleft formation (Figure 1-10). The results supported the notion that collagenase inhibitor affected the collagenase acting on the interstitial collagens and changed the fibrous architecture at the epithelial-mesenchymal interface (Nakanishi *et al.*, 1986) and demonstrated the role of collagen as a source of constraint probably necessary for cleft formation (Nogawa, 1983). Further experiments have indicated that clefting of the terminal buds begins with the contraction of microfilaments in the peripheral cells, but that deposition of collagen and glycosaminoglycans (GAGS) in the mesenchyme at the points of the clefts is required for the subsequent division of the bud (Grobstein and Cohen, 1965).

Indications are that both types I and III collagen synthesis and deposition are required for salivary gland branching morphogenesis (Spooner and Faubion, 1980). But some questions about the absolute requirement for type I collagen in salivary gland morphogenesis remain (Kratochwil *et al.*, 1986). By using submandibular glands of collagen I-deficient Mov-13 mutant mouse embryos, Kratochwil *et al.* demonstrated normal morphogenesis at the 13-day stage. However, based on the work of Spooner and Faubion (1980), and more recent data from Fukuda and associates (1988), it appears that branching morphogenesis is affected by both types I and III collagen.

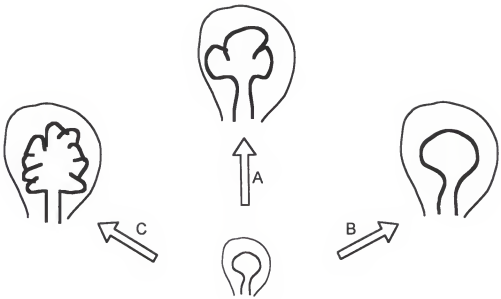


Figure 1-10. Effects of collagenase and collagenase inhibitors on submandibular gland development. Schemiatic summary of epithelial morphogenesis of a mid-day 12 mouse submandibular gland under various conditions. A) cultured in normal media; B) plus 5 μg/ml Clostridial collagenase; and C) plus 5 μg/ml bovine pulp collagenase inhibitor (TIMP). Adapted from Nakanishi and Ishii, 1989.

The convergence of the observations suggesting the importance of other collagens and glycosaminoglycans in the regulation and stabilization of salivary gland morphogenesis came from data indicating that types I and III collagen appear to protect the basal lamina from breakdown by hyaluronidase and other enzymes by binding with heparan sulfate proteoglycan. This binding stabilizes the basal lamina and reduces the breakdown of the heparin-rich proteoglycan (Bernfield *et al*., 1984; Spooner and Faubion, 1980) which is the dominant proteoglycan of the maturing salivary epithelial basal lamina (Cutler *et al*., 1987). Deposition of type III collagen at the cleft or branch point areas seem to play a key role in regulating both assembly of basal lamina components and stabilizing these points so that branching may proceed.

The distribution of ECM macromolecules during development has been examined in various systems including whole mouse embryos (Leivo *et al*., 1980), mouse salivary glands (Bernfield *et al.,* 1972; Banerjee *et al.*, 1977; Spooner, *et al.,*1985, 1986, 1989) and rat submandibular glands (Kadoya and Yamashina, 1989). These initial studies, using fixed or frozen sections, provided valuable information concerning the spatial and temporal distribution of ECM macromolecules during embryogenesis. Hardman and Spooner used confocal microscopy to localize BM-1 proteoglycan, fibronectin, laminin, and collagen types I, IV, and V in developing mouse submandibular and sublingual glands in 3-D (Hardman and Spooner, 1992). Each of the ECM components measured was shown to have a distinct distribution and that the distribution of some of the molecules changed with culture time. Laminin was mainly restricted to the basement membrane. BM-1 proteoglycan was concentrated in the basement membrane and also

formed a fine network throughout the mesenchyme. Type IV collagen was mainly located in the basement membrane of the epithelium, but it was also present throughout the mesenchyme. Type V collagen was distributed throughout the mesenchyme at 24 hours, but at 48 hours was principally located in the basement membrane. Type I collagen was distributed throughout the mesenchyme at all culture times and accumulated in the clefts and particularly at the epithelial-mesenchymal interface as time in culture increases. Fibronectin was observed throughout the mesenchyme at all times.

One of the most interesting questions in animal developmental biology is how an epithelium changes its shape. Nakanishi and Ishii have proposed a model using mouse submandibular glands to describe how several organs including the salivary glands, pancreas, lung, kidney, and mammary glands produce elaborate epithelial structures with maximal surface area (Nakanishi and Ishii, 1989). The results from Nakanishi and Ishii (1989) suggest that collagen may modulate branching morphogenesis of submandibular epithelium in such a way that the number of clefts formed depends on the amount of some type of collagen present in the tissue. Bundles of collagen fibrils were visualized at the narrow clefts between two adjacent epithelial lobules, consistent with the observations of Bernfield and Wessells (1970). These studies raised the question of which specific collagen molecule is important for the morphogenetic change. Earlier work had shown that the most abundant interstitial collagen in submandibular glands is collagen I.

Further work by Nakanishi *et al.* (1988) using antibodies specific to various types of collagen demonstrated strong collagen III reactivity in the very early clefts of late day 12 and early day 13 mouse submandibular glands. Staining for collagen I showed a

rather uniform distribution both at the epithelial-mesenchymal interface and throughout the mesenchyme. Collagen IV was uniform in the basal lamina. These results demonstrated that collagen III, which is known to be relatively rich in embryonic tissues, may play an important role in submandibular gland morphogenesis. With this information, Nakanishi proposed a working model on how the mesenchymal cells secrete collagen which exerts mechanical force to the epithelium giving strain to the surface of the epithelial lobule and initiating cleft formation.

The role of cell proliferation, cell shape change and extracellular matrices in branching morphogenesis has been investigated in the branching morphogenesis of the fetal mouse submandibular gland. However all the studies were based on organ culture experiments whereby the epithelium and mesenchyme interacted as easily as *in situ.* Takahashi and Nogawa (1991) introduced a more analytical experimental system by examining epithelial branching on Matrigel®, a commercially available gel composed of ECM components derived from cultured cells. In those studies the submandibular epithelium showed fair growth and branching morphogenesis when clotted with Matrigel® and separated from the mesenchyme by a porous filter demonstrating that the Matrigel® provided a good extracellular environment for epithelial growth. In addition the epithelium clotted with Matrigel® with mesenchyme on the opposite side of the filter shows far better growth than the one without mesenchyme indicating that mesenchymal factors stimulate epithelial growth.

The temporal and spatial expression of laminin, collagen types IV and I and $\alpha 6/\beta 1$ integrin receptor (the major laminin receptor of the integrin family) has been

examined in the postnatally developing rat parotid gland (Lazowski *et al.*, 1994). Laminin B1 and B2 isoforms and collagens α1(IV) and α1(I) steady-state mRNA levels were highest at ages 0, 7, and 14 days after birth and declined to the adult (90 days) level at 21 days and older. Laminin A chain transcripts were not detected at any age. Collagen α1(IV) and laminin were localized in the basal membrane of the developing acinar and ductal cells, while collagen α1(I) was localized in the stroma surrounding the cells. The amounts of these ECM components were high at the early stages of development and lower at later times. The pattern of expression for the α6/β1 integrin genes during development was similar to those of laminin and collagen α1(IV) and α1(I). These data suggested that laminin B1 and B2 and collagen α1(IV) and α1(I) are consistently coexpressed with the α6/β1 integrin receptor complex in the developing acinar and ductal cells during parotid postnatal development in the rat.

Through studies such as these the importance of the extracellular matrix in the development of salivary glands has been demonstrated, but because of the limited tissues available, no attempt has yet been made at quantifying the ECM gene expression in developing salivary glands.

Contribution of Glycosaminoglycans in Salivary Gland Morphogenesis

The potential importance of glycosaminoglycans in the regulation of salivary gland morphogenesis was first suggested by Kallman and Grobstein (1966). It was suggested that, in addition to collagen, the epithelial basal lamina might be important in the regulation of salivary gland morphogenesis. The basal lamina of the embryonic

submandibular gland is an extracellular product of the epithelium (Banerjee *et al.*, 1977), which is rich in proteoglycan and hyaluronic acid (Cohn *et al.*, 1977) and additionally has been reported to contain type IV collagen, laminin, fibronectin, and chondroitin sulfate and heparan sulfate proteoglycans (Bernfield *et al.*, 1984). That the branching morphogenesis of the epithelial component of developing salivary glands is maintained by its basal lamina has been demonstrated (Banerjee *et al.*, 1977; Bernfield and Banerjee, 1982; Bernfield *et al.*, 1972; Cohn *et al.*, 1977). A number of studies have demonstrated that it is the proteoglycan associated with the basal lamina that play a major role in the regulation and maintenance of the characteristic branching pattern of salivary glands (Bernfield *et al.*, 1984; Cutler, 1989; Spooner *et al.*, 1986).

During early morphogenesis of the mouse and rat submandibular gland xylose-linked proteoglycan is the predominant proteoglycan synthesized by the growing salivary epithelium (Banerjee *et al.*, 1977; Cohn *et al.*, 1977; Cutler *et al.*, 1991a). The direct proof that basement membrane xylose-linked proteoglycans were of critical importance in the control of salivary gland branching morphogenesis came from studies by Spooner and coworkers (Thompson and Spooner, 1982, 1983; Spooner *et al.*, 1985). In these studies they used *p*-nitrophenyl-β-D-xylopyranoside (β-D-xyloside), an inhibitor of proteoglycan assembly, in *in vitro* experiments to provide direct evidence that blockage of proteoglycan assembly inhibited submandibular gland morphogenesis. Both chondroitin sulfate and heparan sulfate proteoglycan have the same (ser-xyl-gal-gal-) linkage region between the appropriate GAG molecules and their respective core proteins, rendering the

synthesis of both proteoglycan types sensitive to exogenous β-D-xylosides (Thompson and Spooner, 1983).

Data which showed the significant role of the basal lamina and its components was developed by Bernfield and his colleagues (Banerjee *et al.*, 1977; Bernfield and Banerjee, 1972; Cohn *et al.*, 1977). Glycosaminoglycans (proteoglycans) and type I collagen were shown, by histochemical, electron microscopic, and biochemical studies, to be concentrated in the clefts or branch points while these molecules appeared to be absent or sparsely distributed at the growing ends of the branches (Banerjee *et al.*, 1977; Bernfield *et al.*, 1972; Bernfield and Banerjee, 1972; Cohn *et al.*, 1977).

Autoradiographic studies suggested that there were regional differences in glycosaminoglycan turnover and that there was an increased degradation of the basement membrane which occurred at the growing end of the branches. This degradative activity seemed to be mediated by the investing connective tissue since there was an activation of a neutral hyaluronidase with specificity for hyaluronic acid and chondroitin sulfate proteoglycan in the tissue at the time branching morphogenesis was initiated. (Bernfield and Banerjee, 1982; Bernfield *et al.*, 1984). Coincident with the activation of this hyaluronidase, the pattern of glycosaminoglycans produced by the salivary epithelium changed. The glycosaminoglycans synthesized by the early rudiments were rich in hyaluronic acid and chondroitin sulfate. However, as morphogenesis progressed and became more complex the glycosaminoglycans produced showed an increased amount of heparin sulfate and a reduced content of hyaluronic acid and chondroitin sulfate (Banerjee *et al.*, 1977; Cohn *et al.*, 1977; Cutler *et al.*, 1991b). In the mature gland, heparan sulfate

is the predominant glycosaminoglycan synthesized with only small amounts of hyaluronic acid and virtually no chondroitin sulfate produced (Cutler *et al.*, 1987).

Collectively these data, along with definition of distinct patterns of proteoglycan deposition that correlates with events of lung development (Smith *et al.*, 1990), strongly suggest a role for glycosaminoglycans, specifically chondroitin sulfate proteoglycan, in the morphogenetic branching of salivary glands. However, neither the localization of individual proteoglycans nor definition of their roles in the morphogenic changes is yet defined (Vogel, 1994).

Salivary Gland Cytodifferentiation

Information regarding the molecular factors involved in the regulation of secretory cell differentiation in the salivary glands is not as advanced as that for salivary gland morphogenesis. The basic morphogenetic branching pattern is established prior to the initial events in secretory cell development (Cutler, 1980). As the branching pattern of the rudiment is being established, cells producing low levels of secretory proteins can be identified within the rudiment. However, these cells do not demonstrate any structural signs of differentiation such as secretory granules at this time (Cutler, 1973; Cutler and Chaudhry, 1974; Yamashina and Barka, 1973). By the time secretory granules, the first fine structural sign of secretory cell differentiation, can be seen in developing secretory cells, branching morphogenesis is very advanced (Yamashina and Barka, 1972, 1973,

1974; Redman and Sreebny, 1970b, 1971; Redman and Ball, 1978; Cutler and Chaudhry, 1973a, 1973b, 1974).

Role of Epithelial-Mesenchymal Interactions in Salivary Gland Cytodifferentiation

Direct epithelial-mesenchymal contact between the salivary epithelium and the surrounding capsular mesenchyme (Cutler and Chaudhry, 1973a, 1973b; Cutler, 1977) appears to have an important role in initiating the sequence of events (amplification of secretory protein synthesis and the subsequent structural changes at the cellular level) that lead to secretory cell cytodifferentiation within the salivary glands (Cutler, 1980). These contacts are seen only after the primary branching pattern has been established and only involve interaction between the epithelial cells in the end-buds of the initial 4 to 12 branches of the early rudiment and the surrounding mesenchyme (Cutler, 1977; Cutler and Chaudhry, 1973b). Contacts of this type are not seen before or after this stage of development of the gland. If these direct epithelial-mesenchymal interactions are prevented by separating the developing epithelium from its investing mesenchyme differentiation of secretory cells does not occur. However, if the epithelial-mesenchymal contacts have taken place, many of the cells go on to develop into secretory cells (Cutler, 1980).

The continued presence of the mesenchyme does not seem to be required for secretory cell development to occur once the inductive signals for secretory cell differentiation have been generated. This observation points out a significant difference between morphogenesis of the rudiment and secretory cell development, since the

continued presence of the mesenchymal capsule is required in order for morphogenesis to occur. In addition the attainment of apical-basal polarity of the secretory cells seems to require factors produced by the mesenchyme. Secretory cells differentiating in the absence of the capsular mesenchyme produce secretory material and package the material into secretory granules. However, the cells do not demonstrate the apical-basal polarity typical of salivary gland exocrine cells. Further, the clustering of cells into acinar units is not required for the differentiation of secretory cells. These observations suggest that the development of the typical glandular morphogenetic branching pattern and the differentiation of secretory cells within developing salivary lands are partially coupled but independently regulated processes (Cutler, 1980). The mesenchyme seems to regulate both the gross, glandular morphology and the fine, cytological form (columnar shape, apical-basal polarity) of the individual cells within the gland.

Contribution of the Extracellular Matrix in Salivary Gland Cytodifferentiation

It was noted that there was a fibrillar material between the opposing membranes of the direct epithelial-mesenchymal contacts involved in secretory cell differentiation (Cutler, 1977; Cutler and Chaudhry, 1973b). The fibrillar material was structurally similar to the lamina densa of the basal lamina and suggested the possibility for a role for extracellular matrix molecules in the regulation of secretory cell differentiation.

Recent studies (Cutler, 1990) have examined the effects of culturing developing 16-day embryonic rat submandibular gland rudiments in the presence or absence of polyclonal or monoclonal antibodies directed against extracellular matrix components.

These studies have provided some interesting insights into the regulation of glandular morphogenesis and secretory cell differentiation It appears that type IV collagen and laminin have roles in the regulation of salivary gland morphogenesis in addition to the roles already established for types I and III collagen and chondroitin sulfate proteoglycan. To date, with regard to the control of the differentiation of salivary gland secretory cells, type IV collagen is the only extracellular matrix molecule which was shown to play a role in this process. When embryonic submandibular gland rudiments are cultured in the presence of anti-type IV collagen antibody virtually all of the rudiments were prevented from undergoing further branching morphogenesis and secretory cell differentiation was not observed in any of these rudiments.

The processes of morphogenesis and cytodifferentiation are partially linked, independently regulated processes. The full expression of both processes is modulated or controlled by epithelial-mesenchymal interactions involving both direct contacts and indirect molecular interactions. It appears that, at least in part, molecular constituents of the extracellular matrix may mediate the regulatory effects of both direct and indirect interactions. Data indicate that epithelial-mesenchymal interactions and various extracellular matrix molecules are intimately involved in the induction, control, and maintenance of salivary gland morphogenesis and cytodifferentiation.

Contribution of Glycosaminoglycans in Salivary Gland Cytodifferentiation

Previous studies (Bottaro and Cutler, 1984; Cutler, 1980; Cutler *et al.*, 1985) suggested that the process of differentiation of salivary gland secretory cells is controlled

independently from the process of glandular morphogenesis. To test this, Cutler *et al.,* examined the role of xylose-linked proteoglycans, molecules known to play a significant role in the regulation of salivary gland morphogenesis, in the control of secretory cell differentiation in developing salivary glands (Cutler *et al.*, 1991b). In these studies 16-day rat embryonic submandibular gland rudiments were cultured in the presence or absence of β-D-xyloside under conditions known to permit secretory cell differentiation. Morphogenesis was inhibited in greater than 90% of the rudiments cultured in the presence of xyloside analogous to the findings of Spooner and coworkers (Spooner *et al.*, 1985). However, secretory cell differentiation was seen in more than 70% of the rudiments in which branching morphogenesis had been inhibited. Analysis of the effect of β-D-xyloside on glycosaminoglycan/proteoglycan synthesis by rat salivary gland rudiments indicated that xyloside specifically inhibited chondroitin sulfate synthesis but had little effect on heparan sulfate proteoglycan production. This observation is consistent with those of Spooner and coworkers (Spooner *et al.*, 1986) and earlier reports by Thompson and Spooner (1982, 1983) which indicated that chondroitin sulfate proteoglycan plays a major role in the regulation of salivary gland morphogenesis.

Proteoglycans play a very central role in the regulation of the morphogenesis of salivary glands. Chondroitin sulfate proteoglycan clearly appears to be the major proteoglycan in this regard. Interestingly, chondroitin sulfate proteoglycan does not appear to be involved in the regulation of secretory cell differentiation within the developing rudiment. The epithelial cells also secrete GAGS and other ECMs such as fibronectin and laminin, which strongly influence the course of cytodifferentiation in the

gland (Bernfield *et al.*, 1984). This observation supports earlier work (Cutler, 1980) that indicated that morphogenesis and cytodifferentiation of salivary glands are separately regulated processes.

Statement of the Problem

The preceding discussion of salivary gland morphogenesis and cytodifferentiation shows the great historical interest in salivary gland development and describes compelling evidence that the extracellular matrix is critical to the development of salivary glands. While the literature supports the important role of the extracellular matrix in salivary gland development there is complete lack of ECM gene quantitation accompanying this data. Until recently the ability to measure gene expression from small developing tissues was not possible, but with the advent of polymerase chain reaction (PCR) technology, it is now possible to begin to understand the changes that occur in gene expression in developing tissues. The research project in this dissertation reports on the development of a multiprimer supertemplate to measure gene expression of ECM genes. The technology is so powerful that it permits quantitative gene expression from small developing mouse submandibular gland rudiments. The following section will describe the history of PCR technology that has led to competition-based quantitative RT-PCR, one of the techniques used in this report.

Gene Quantitation

The disadvantage of working with fetal samples, particularly early embryos or dissected fetal organs, is that it is often difficult to obtain sufficient tissue for many molecular analyses. Sensitive methods commonly used for the detection and analysis of RNA molecules include *in situ* hybridization, Northern gels, dot or slot blots, S1 nuclease studies, and RNase A protection studies (Kawasaki, 1990). The most sensitive of these methods is *in situ* hybridization, in which 10 to 100 molecules can be detected in a single cell. However, *in situ* hybridization is technically very difficult and does not lend itself to the processing of large number of samples. With other techniques the level of detection is approximately 0.1 to 1.0 pg of the target sequence which for an average-sized mRNA translates to 10^5 to 10^6 target sequence molecules. The high sensitivity of RT-PCR overcomes this limitation. The first published description of the use of PCR to amplify mRNA from cDNA was in 1987 (Veres *et al.*, 1987), when it was used to study point mutations in the mouse ornithine transcarbamylase gene.

The use of RT-PCR quickly turned toward quantitation of gene expression. Initially many groups attempted to coamplify a gene of interest in comparison to the amplification of a housekeeping gene, *i.e.,* β-actin, glyceraldehyde-3-phosphate dehydrogenase, keratins, *etc.* These coamplifications were done in either separate tubes or in a single tube, but when RNA is limited, it is desirable to amplify both the test and reference mRNAs in a single tube as long as the two products are of different sizes. Braga and Gendler (1994) found however, that the yield of muc-1, an epithelial

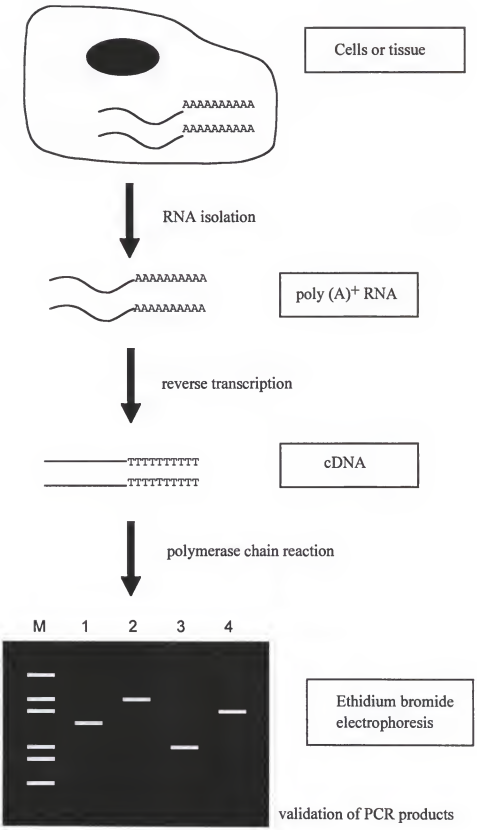
differentiation marker in mouse embryos, and β-actin can vary if they are amplified together in a single tube. The ratios of Muc-1 to β-actin also differed when amplified individually or together even with the use of gene-specific primers in the RT reaction. This led to the conclusion that a more sophisticated system was needed to simultaneously amplify more than one cDNA in one single reaction.

RT-PCR

Detection of low abundance mRNAs by reverse transcription-polymerase chain reaction (RT-PCR; Figure 1-11) has become a standard technique to study gene expression in tissues and cells in culture. The ability to determine relative or absolute copy number of specific mRNAs has been difficult however due to inadequate internal standards to control for sample-to-sample variation (Tarnuzzer *et al.*, 1996a). The use of a synthetic RNA standard with identical sequences to the PCR primers allows reproducible quantitation between samples and assays. By designing multisequence templates, specific mRNAs can be quantified using a single template. Addition of multiple templates to a single RT reaction allows the quantitation of large number of targets from as little as 1 μg of total RNA. Quantitative RT-PCR therefore provides a very powerful technique to study gene expression in tissues which are limited due to size or availability

One of the applications of PCR is the quantitative analysis of specific nucleotide sequences. The main advantage of this method, as compared with other methods based on probe hybridization, is its extreme sensitivity and specificity. However, because of the

Figure 1-11. Schematic diagram of the RT-PCR method. Briefly, either total or poly(A)$^+$ RNA is isolated whole tissue or cells. The RNA is then reverse-transcribed to make cDNA using either oligo (dT), random hexamer, or gene-specific primers. The cDNA is amplified by PCR. The products are separated on an agarose gel, stained with ethidium bromide, and photographed.


Cells or tissue
AAAAAAAAAA
AAAAAAAAAA
RNA isolation
AAAAAAAAAA
AAAAAAAAAA
poly (A)+ RNA
reverse transcription
TTTTTTTTTT
TTTTTTTTTT
cDNA
polymerase chain reaction
M 1 2 3 4
Ethidium bromide electrophoresis
validation of PCR products

many amplification steps, PCR by itself is not an accurate quantitative assay. Small differences in amplification efficiency can result in dramatic differences in product yield. A number of controls have been introduced to compensate for the problems intrinsic to quantitation using PCR. The use of PCR for gene quantitation has been attempted a number of times in the literature, but because of the intrinsic problems in PCR amplification, sufficient controls have not been included to overcome serious concerns with the methodology.

Competitive RT-PCR

The name "competitive PCR" refers to PCR that is allowed to proceed into the saturation phase in which a competition occurs between target and standard templates for available substrates. However, because the sum of the masses of both products cannot exceed some maximum value, the amount of product formed from one template will decrease with the increasing quantity of the other template. A related quantitative application of PCR coamplification is the determination of relative mRNA levels of members of gene families. The term "ratio PCR" has been suggested for this method. Instead of housekeeping genes, many investigators have used exogenously added standards. The conditions for reliable quantification are much less stringent if the standard and target sequences possess identical primer binding sites. The use of an exogenously added standard that contains the same sequences for primer binding as the target was first described by Wang *et al.* (1989). This technique was also explored by Becker-Andre, who named the method PATTY: PCR-aided transcript titration assay (1993), and by Gilliland *et al.,* who introduced the term "competitive PCR" (Gilliland *et al.*, 1990). This

latter term was adopted in most of the literature. Because only one primer pair is needed in this approach, it can be assumed that the amplification efficiency is identical for both sequences if the region in between the primer-binding sites also is very similar. Therefore, the PCR can be extended into the latent phase where a decrease of the efficiency occurs, if this decrease occurs with an identical time course for both sequences. The ability to extend the PCR into the nonexponential phase is very useful in practice because it avoids many additional controls and allows accurate quantification of the PCR products (Raeymaekers, 1995).

In competitive PCR, the products of target and standard sequences are discriminated either by a difference in length or by a specific restriction site in the region between the primer templates. In practice, a series of PCR tubes containing the same but unknown amount of target sequence is spiked with a dilution series of defined quantities of the standard. If the rate of amplification is the same for both sequences, their ratios will remain constant during the amplification and the amount of the unknown template can be quantitated from the ratio of the two products. In the practical application of the method, it was recommended that a curve be constructed relating the logarithm of the ratio of PCR products (standard/target) to the logarithm of the initial copy number of standard template added. The amount of initial target template can be read easily from the point on the curve where the amounts of target or RNA and standard or template are equal (Siebert, 1993; Siebert and Larrick, 1992).

Although competitive PCR has been used in several examples for reliable quantification, many incorrect results have also been published, as is apparent from the shape of the log-log calibration curves. Theory predicts that this calibration curve should be linear and have a slope of 1. An invalid standard curve must be caused by a difference in the amplification factor

between the template and the standard, or by differences in the PCR tubes of the dilution series (Raeymaekers, 1994).

For the analysis of submandibular gland ECM gene expression, a "thematic" multiprimer supertemplate, pMATRIX, has been constructed and used to measure the gene expression for 7 ECM genes by competition-based quantitative RT-PCR. It represents the first report of quantitative gene expression in developing submandibular glands. To correlate with this data, immunohistochemistry was performed on developing mouse submandibular glands to localize the ECM proteins.

CHAPTER 2
MATERIALS AND METHODS

Synthetic Supertemplate pMATRIX Construction

To investigate the changes in extracellular matrix gene expression during fetal submandibular gland development, a multiprimer plasmid (pMATRIX) was constructed which contains primer sequences for collagens α2(I), α1(III), and α1(IV), elastin, fibronectin, laminins B1 and B2, and lysyl oxidase. The multiprimer sequence with an added poly(A) tail was inserted into pCRII downstream from an Sp6 RNA polymerase promoter.

Selection of oligonucleotide sequences. The nucleotide sequences for the human, mouse and rat ECM genes were retrieved from Genbank®. The primer sequences selected were 100% identical for all three species (Table 2-1) and were selected to amplify polymerase chain reaction (PCR) products between 500-800 base pairs. In addition, oligonucleotide primers, also 100% identical between human, mouse, and rat, were synthesized to correspond to internal sequences approximately 50 bp downstream from either the 5' or 3' primers. Appendix A lists the code names for each of the ECM oligonucleotide primers. The oligonucleotide primers were also designed to span introns where possible to control for the amplification of contaminating genomic DNA.

Table 2-1
List of ECM genes with their respective upstream (5'), downstream (3'), and internal oligonucleotides with the expected size of the pMATRIX template and cellular mRNA amplicons

	Primers			Size (bp)	
gene	**5'**	**3'**	**internal**	**plasmid**	**mRNA**
fibronectin	CCGGGTTCTGAGTACACAGTC	GGAGGGTCTCTTCACCAGGGA	GCCAGCCCCTGATTGG	347	768
laminin B2	GGGCAGCCTTCCTGACCGACT	GGAAAGAAGTTACTGGCCGGC	GCTCCAAACCGCCTTA	344	677
lysyl oxidase	GGGAGAACAACGGGCAGGTGT	GGCTGGGGATGATGTAGGTCC	CACTGGTTCCAAGCTG	340	528
collagen α1(III)	GGCTCCTGGTGAGCGAGGACG	CCTCTTGGACCACGTTTACCC	CCTGGATCCCCTGGTG	338	522
elastin	TGCAGGCTTAGTGCCTGGTGG	CTCGACGTCGACCCGAACCACG	CCGACGGTTTCGGGTC	338	704
collagen α2(I)	GGCCCTGGCACCAATGGGCTTA	CCACGGTCTCCTGAACAACCA	GGCACCACTTCACCCA	335	769
collagen α1(IV)	GGACAAGCAGGCTTTCCTGGA	GGGACCGGAAGGACCTGTCGT	CCTGGATCCCCTGGTG	335	743
laminin B1	CTGCGCAGAAGGCAGCTGCTA	TCACCTTCCTTACCAAGTGCC	GCTCCAAACCGCCTTA	335	594

FN-3' CCTCCCAGAGAAGTGGT**CCT**

LAMB2-3' **CCT**TTCTTCAATGACCGG**CCG**

LO-3' **CCG**ACCCCTACTACATCCA**GG**

COL3A1-3' **GG**AGAACCTGGTGCAAATGG**G**

ELAST-3' **G**AGCTGCAGCTGGGCTT**GGTGC**

COL1A2-3' **GGTGC**CAGAGGACTTGTTGGT

COL4A1-3' CCCTGGCCTTCCTGGACAGCA

GTAK-3' GTGAAGGAGACGGGCTTCC**A**

LAMB1-3' **A**GTGGAAGGAATGGTTCACGG

LAMB2-3'
CCTCCCAGAGAAGTGGT**CCT**TTCTTCAATGACCGG**CCG**ACCCCTACTA
FN-3' LO-3'

COL3A1-3'
CATCCA**GG**AGAACCTGGTGCAAATGG**G**AGCTGCAGCTGGGCTT**GGTG**
ELAST-3'

COL1A2-3' GTAK-3'
CCAGAGGACTTGTTGGTCCCTGGCCTTCCTGGACAGCACCTGAAGGAG
COL4A1-3'

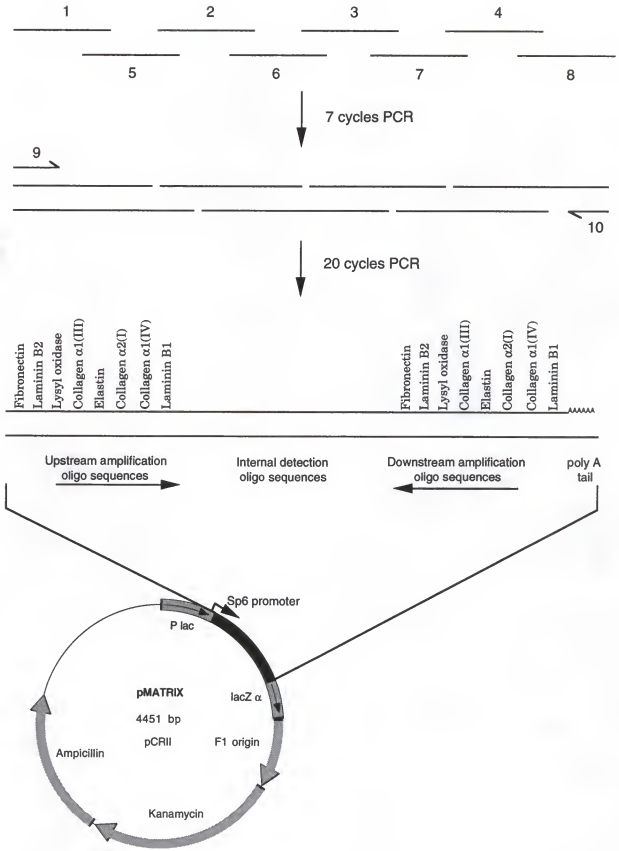
ACGGGCTTCC**A**GTGGAAGGAATGGTTCACGG
LAMB1-3'

Figure 2-1. Alignment of oligonucleotides to create the 501 bp multiprimer insert. The most economical arrangement was made as shown above for the 3' primers.

Synthesis of multiprimer template. The multisequence insert was synthesized by PCR using overlapping 80-mer oligonucleotide primers (Tarnuzzer *et al.*, 1996a) containing contiguous ECM gene sequences. Before the 80-mers were synthesized, however, the selected 5' and 3' oligonucleotide primers were tested for specificity using standard RT-PCR using a tissue which is known to express the ECM genes. Total RNA from mouse liver and placenta were used to test these primers. Amplification of a single band of the correct size (confirmed by restriction endonuclease digestion) indicated a good primer pair had been selected and synthesized. Amplification of multiple bands [as is the case for collagen α1(III)] was considered acceptable if a temperature was found which reduced the other bands. Primers which produced a non-specific product(s) with a molecular weight similar to the correct product were excluded for use in the plasmid. Each of the 5' oligonucleotide primer sequences was aligned and examined for common overlapping ends. The same was done for the 3' oligonucleotides. The arrangement that was the most economical, *i.e.,* had the most overlaps in the 5' and 3' primers, was the sequence used to determine the order of the genes arranged on the plasmid. An example of how this was achieved in shown in Figure 2-1. For pMATRIX, the most economical arrangement of the oligonucleotide primers was 501 bp. A restriction map of the insert is shown in Appendix B. From this sequence eight overlapping 80-mers were then selected and synthesized by the University of Florida DNA Synthesis Laboratory.

The construction of pMATRIX is diagramatically represented in Figure 2-2. To synthesize the multisequence insert, 500ng of each 80-mer oligonucleotide was added in a 100µl standard PCR run with 200 µM each dNTP, 2.5U *Taq* polymerase in a buffer

Figure 2-2. Scheme for construction of pMATRIX. The 8 overlapping synthetic oligonucleotides were ligated and filled with *Taq* polymerase in a multi-step process as described. The compete template sequence was unidirectionally cloned into plasmid pCRII to allow cRNA synthesis by Sp6 RNA polymerase (Tarnuzzer *et al.,* 1996). GTAK = GTA kinase (p58), a unrelated gene included on this plasmid.


1
2
3
4
5
6
7
8
7 cycles PCR
9
10
20 cycles PCR
Fibronectin
Laminin B2
Lysyl oxidase
Collagen α1(III)
Elastin
Collagen α2(I)
Collagen α1(IV)
Laminin B1
Fibronectin
Laminin B2
Lysyl oxidase
Collagen α1(III)
Elastin
Collagen α2(I)
Collagen α1(IV)
Laminin B1
Upstream amplification oligo sequences
Internal detection oligo sequences
Downstream amplification oligo sequences
poly A tail
Sp6 promoter
P lac
pMATRIX
4451 bp
pCRII
lacZ α
F1 origin
Ampicillin
Kanamycin

containing 50 mM KCl, 10mM Tris, pH 8.0 and 1.5 mM $MgCl_2$. This reaction was amplified for 1 cycle at 94°C for 7 min for denaturing, 55°C for 2 min for annealing, and 72°C for 3 min for extension, followed by 7 cycles of 94°C for 1.5 min, 55°C for 2 min, and 72°C for 3 min, and finally incubated at 4°C indefinitely. To add the flanking oligonucleotides, a second polymerase chain reaction was performed consisting of 2µl of the product from the above polymerase chain reaction as well as the 5' and 3' oligonucleotide primers (primers 9 and 10 in Figure 2-2). The entire multisequence insert was then amplified by PCR for 25 cycles at 94°C for 1.5 min, 55°C for 2 min, and 72°C for 3 min. The resulting 501 bp reaction product was separated on a 2% low melting point (LMP) agarose gel and the band excised for cloning into pCRII. Figure 2-3 is the sequence of the 501bp multisequence insert. pCRII is a commercially available plasmid from Invitrogen (San Diego, California) and is contained in the TA Cloning kit (K2000-01). pCRII contains both an Sp6 and a T7 RNA polymerase promoter which flanks a polylinker site designed to easily accept PCR generated products by nature of a 5' adenine overhang.

Ligation. The low melting point agarose gel slice containing the multisequence insert was heated to 65°C for 10 minutes and then kept at 37°C for the subsequent ligation reaction (Sambrook *et al.,* 1989). The ligation reaction consisted of 1 µl of the PCR product (approx. 10ng), 6 µl of sterile water, 25 ng of pCRII (freshly resuspended in 8.8 µl of Tris/EDTA buffer), 1 µl of T4 ligase (Invitrogen), and 1 µl of 10X ligation buffer (60mM Tris pH 7.5, 60mM $MgCl_2$, 50mM NaCl, 1 mg/ml BSA, 70mM βME, 1mM ATP, 20mM DTT, 10mM spermidine). The reaction was incubated at 12°C overnight.

```
  1  CCGGGTTCTGAGTACACAGTCGGGCAGCCTTCCTGACCGACTGGGAGAAC
     GGCCCAAGACTCATGTGTCAGCCCATCGGAAGGACTGGCTGACCCTCTTG

 51  AACGGGCAGGTGTGGCTCCTGGTGAGCGAGGACGTGCAGGCTTAGTGCCT
     TTGCCCGTCCACACCGAGGACCACTCGCTCCTGCACGTCCGAATCACGGA

101  GGTGGCCCTGGACCAATGGGCTTAGGACAAGCAGGCTTTCCTGGATGCCA
     CCACCGGGACCTGGTTACCCGAATCCTGTTCGTCCGAAAGGACCTACGGT

151  GGGGAGGTGAAGACCCTGCGCAGAAGGCAGCTGCTAGCCAGCCCCTGATT
     CCCCTCCACTTCTGGGACGCGTCTTCCGTCGACGATCGGTCGGGGACTAA

201  GGTAACTGCCACTGACACTGGTTCCAAGCTGCCTGGATCCCCTGGTGGCT
     CCATTGACGGTGACTGTGACCAAGGTTCGACGGACCTAGGGGACCACCGA

251  GCCAAAGCCCAGCCGTGGTGAAGTGGGTCAGGGGATCAGAGGTGCATGTT
     CGGTTTCGGGTCGGCACCACTTCACCCAGTCCCCTAGTCTCCACGTACAA

301  CCCCACGTGGGCTCCAAACCGCCTTACCTCCCAGAGAAGTGGTCCCTTTC
     GGGGTGCACCCGAGGTTTGGCGGAATGGAGGGTCTCTTCACCAGGGAAAG

351  TTCAATGACCGGCCGACCCCTACTACATCCAGGAGAACCTGGTGCAAATG
     AAGTTACTGGCCGGCTGGGGATGATGTAGGTCCTCTTGGACCACGTTTAC

401  GGAGCTGCAGCTGGGCTTGGTGCCAGAGGACTTGTTGGTCCCTGGCCTTC
     CCTCGACGTCGACCCGAACCACGGTCTCCTGAACAACCAGGGACCGGAAG

451  CTGGACAGCACCTGAAGGAGACGGGCTTCCAGTGGAAGGAATGGTTCACG
     GACCTGTCGTGGACTTCCTCTGCCCGAACCTCACCTTCCTTACCAAGTGC

501  G
     C
```

Figure 2-3. Nucleotide sequence of the 501 bp multiprimer insert.

Transfection. Fresh Luria broth plates with 50μg/ml ampicillin were prepared as per (Sambrook *et al.,* 1989) for the transfection. Competent cells (INVαF') were incubated at 4°C for 30 minutes with 2 μl of beta-mercaptoethanol (βME) and 1 μl of the ligation mixture. The cells were immediately heat-shocked at 42°C for 30 sec and then further incubated at 4°C for 2 min. To this vial 450μl of warmed SOC medium was added and incubated at 37°C with shaking at 225 rpm. While the cells were shaking, 25 μl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 40mg/ml) was spread on the Luria broth/ampicillin plates. Either 25 or 100μl of the transfection reaction mixture was spread on the LB/ampicillin plates, inverted and incubated overnight.

The pCRII has a number of added advantages including classical blue/white selection due to the inclusion of the *lacZ*α complementation fragment as well as ampicillin and kanamycin resistance. All colonies of the transfected bacteria which grow on the plates have been conferred ampicillin resistant through the uptake of the pCRII plasmid. Bacterial colonies which contain pCRII with no insert will be blue while colonies containing pCRII with the insert will be white. The white colonies (and some blue colonies to use as negative controls) were selected for alkali lysis mini-preps.

Determination of positive clones. To determine which of the clones were positive for the 501 bp insert, the colonies were selected as described above, grown up in 5 ml of ampicillin containing LB broth. A mini-plasmid DNA prep (Sambrook *et al.,* 1989) and restriction endonuclease digestions (Sambrook *et al.,* 1989) were performed to identify those clones which contained the multisequence insert. *Nhe*I (New England Biolabs, Boston, MA) cuts the 501 bp multisequence insert once at position 183 and does not cut

pCRII. By performing a double digest with *Hin*dIII, which cuts pCRII in the multiple cloning site at 277 but not the multisequence insert, it was possible to test for both the presence and orientation of the multisequence insert in pCRII. If the insert was not present, digestion with *Nhe*I/*Hin*dIII would yield the linearized plasmid of 4400 bp. If the insert is present and is in the 5' to 3' orientation, digestion with *Nhe*I/*Hin*dIII would yield a restriction digest product of 243 bp. If the insert is present and is in the 3' to 5' orientation, digestion with *Nhe*I/*Hin*dIII would yield a product of 378bp. Digestion with *Eco*R1, which does not cut the insert or the plasmid but does release the insert, can be used to confirm the presence of the insert. Two clones were identified which contained the multisequence insert and were in the 5' to 3' insert and therefore would use the Sp6 RNA polymerase promoter. In addition, PCR amplification with different pairs of oligonucleotide primers included on the plasmid confirmed that the multisequence insert was intact (the 5' fibronectin, MH61 and the 3' laminin B2, MH74 as well as with all nine pairs of 5' and 3' oligonucleotide primers).

Cesium chloride gradient. Preparation of a large maxi-prep from the positive clones (Sambrook *et al.*, 1989) was performed. Briefly, this involved separating the plasmid and cellular DNA on a cesium chloride (CsCl) ethidium bromide (EtBr) gradient. Centrifugation of the CsCl EtBr gradient was done at 45,000 rpm for 24.5 hrs at 4°C using a Beckman VTi50 rotor and L55 ultracentrifuge. The plasmid band was removed from the gradient and collected in 15ml tubes. EtBr was removed through five successive isoamyl alcohol washes until both phases are clear (Sambrook *et al.*, 1989). Finally the residual CsCl was removed from the plasmid DNA by diluting with 3 volumes of sterile

water and 2 volumes of 100% ethyl alcohol (EtOH) and centrifuging 10,000 x g for 15 min at 4°C. The resulting pellet was the dissolved in 0.5 ml TE (pH 8.0), recentrifuged at 10,000 x g as above, washed in 70% EtOH, and finally resuspended in 0.5 ml TE. The concentration of the plasmid was determined by measuring the optical density (OD) at 260nm using a Pharmacia GeneQuant® (Pharmacia, Uppsala, Sweden).

Engineering and Insertion of a Poly(A) Tail

The orientation of the insert was determined by restriction endonuclease digestion. Two oligonucleotides were designed to add the poly(A) tail at the *Xho*I/*Xba*I sites distal to the Sp6 promoter on pCRII (Tarnuzzer *et al.*, 1996a). The oligonucleotide primers retain the *Xho*I and *Xba*I sites as well as add an *Hin*dIII site which allowed for confirmation of insertion by restriction digestion. The two oligonucleotides and the arrangement of the 501 bp insert in relationship to pCRII is shown in Figure 2-4.

Two micrograms of the plasmid were linearized overnight at 37°C with *Xho*I and *Xba*I. The products were separated on a 1.5% LMP agarose gel and the linearized plasmid was excised from the gel and allowed to ligate overnight with the poly(A) tail oligonucleotides. The plasmid was transformed using the TA cloning kit (Invitrogen) as previously described. The transformants were plated on LB/ampicillin plates, however, there is no blue/white color selection here since *lac* is already interrupted by the multisequence insert. To test for the successful insertion of the poly(A) tail, mini-preps were done and the DNA cut with *Hin*dIII. Large scale plasmid isolation was done for the positive clones. The plasmid DNA was purified using the Wizard Maxipreps® DNA

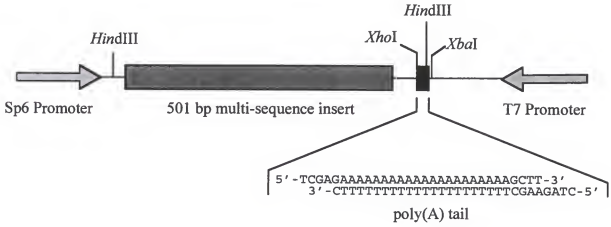


Figure 2-4. Cloning of a poly(A) tail to the multiprimer insert. Oligonucleotide primers were designed and ligated into the *Xho*I/*Xba*I site of pCRII downstream of the multiprimer insert. Digestion with *Hind*III confirmed the ligation of the poly(A) tail.

Purification System (Promega, Madison, WI, A7270) and resuspended in 2 ml TE. Concentration of the plasmid was determined by absorbance at 260nm.

The poly(A)-tail containing plasmid was prepared for the Sp6 transcription reaction (Ausubel, 1987) by digesting the plasmid with *Xba*I and proteinase K. The linearized plasmid was extracted twice with phenol/ chloroform and then precipitated overnight with 2.5 volumes of EtOH at -20°C. The plasmid was centrifuged for 15 min at 14,000 rpm, the pellet washed in 70% (v/v) DEPC/EtOH and resuspended in 30µl DEPC-treated water. The RNA template was synthesized using an RNA transcription reaction containing 5µl of 5x buffer (40mM Tris-HCl pH 7.9, 6mM $MgCl_2$, 2mM spermidine, 10mM dithiothreitol, and 10mM NaCl, Promega), 1µg treated plasmid, 0.5mM ribonucleotides (rNTPs, 1 µl each at 10mM), 20U RNasin, 10U Sp6 (Promega, P1085) and 12µl DEPC-treated water. The 25µl reaction was incubated for 1.75 hr and stored at -20°C.

Isolation of Poly(A)$^+$ RNA

Poly(A)$^+$ RNA was isolated with a Micro-Fast Track mRNA Isolation kit (Invitrogen, K1520-02), a modification of a protocol established by Haqqi *et al.* (1992). Briefly, the Sp6 transcription reaction was diluted up to 1 ml with DEPC-treated water and NaCl was added to 300mM. An oligo-(dT) cellulose pellet was incubated with the RNA and allowed to absorb for 20 minutes at 250 rpm at 37°C. The oligo-dT cellulose slurry with absorbed poly(A)$^+$ RNA was pelleted, resuspended in binding buffer, and loaded onto a spin column. The poly(A)$^+$ RNA was eluted, ethanol precipitated,

resuspended in 10µl DEPC-treated water and quantified by absorbance at 260nm using a GeneQuant® spectrophotometer (Pharmacia).

Sample calculation:

$$0.24\ \mu g/ml\ (OD\ 260nm) \times (\mu mole/214{,}500\ \mu g) = 1.1189 \times 10^{-6}\ \mu moles/ml$$

$$\text{or} = 1.1189 \times 10^{-9}\ \mu moles/\mu l$$

where 214,500 = 650 bases x 330 MW

$$1.1189 \times 10^{-9}\ \mu moles/\mu l \times 6.023 \times 10^{17}\ molecules/\mu mole = \underline{6.73902 \times 10^{8}\ copies/\mu l.}$$

Tenfold serial dilutions of the transcription reaction (10^{-2} to 10^{-9}) were made in DEPC-treated water. Quantitative RT-PCR reactions with different dilutions of template should then give a series of reactions which decrease in intensity with decreasing template copy number.

Isolation of Total Cellular RNA

The method of Chirgwin (Chirgwin *et al.*, 1979) was used for isolating total cellular RNA. Procedures involving experimental animal were performed under protocol number #4206 approved by the Animal Care and Use Committee of the University of Florida. Briefly, timed-pregnant BALB/c mice (days 13 - 18) were sacrificed by cervical dislocation and fetal submandibular glands were excised and lysed in 2 ml of 4M guanidinium isothiocyanate, 25mM sodium citrate (pH 7.0), 0.5% sarkosyl, and 0.1M β-mercaptoethanol, shearing the samples through a 22 gauge needle, and loading the mixture on top of a 2 ml cushion of 5.7M cesium chloride (CsCl), 0.1M ethylenediaminetetraacetic acid (EDTA) in 10mM Tris-HCl. The CsCl gradient was

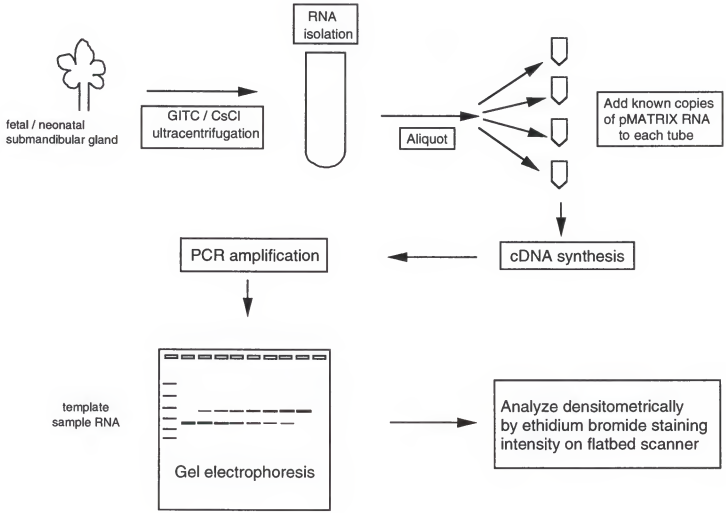
centrifuged at 35,000 rpm in a Beckman SW 50.1 rotor for 18 hours at 20°C. RNA was recovered and stored indefinitely as an ethanol precipitate at -80°C.

Quantitative RT-PCR

The protocol for quantitative RT-PCR (Tarnuzzer *et al.*, 1996a) is shown in Figure 2-5. After the submandibular gland RNA was isolated, cDNA was synthesized in a series of standard 50µl reverse transcription (RT) reactions each containing 1.0µg of total RNA and known dilutions of pMATRIX cRNA ($3.7x10^5$ - $3.7x10^9$ copies), 2.5 µM oligo$(dT)_{16}$, 1.5mM $MgCl_2$, 200 µM dNTP, 50 U/ml human placental ribonuclease inhibitor (Promega), 10mM Tris-HCl (pH 8.3), 50 mM KCl, and 200 U/µg RNA Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Gibco-BRL). The reaction was incubated at 25°C for 10 min, 42°C for 60 min, and 94°C for 5 min.

DNA amplification for each ECM gene was done in standard 50µl polymerase chain reactions which contained 5µl of the RT reaction, 200 µM dNTP, 50 pmoles of each 3' and 5' PCR primer (Table 1), 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50mM KCl, and 0.5µl/reaction *Taq* polymerase (P-E Express, Norwalk, CT; N801-0060). Amplification reactions were carried out in a Biometra thermocycler with 40 sequential cycles at 94°C for 1.5 min, 58°C for 2 min, and 72°C for 3 min, followed by 10 min at 72°C for final extension. The 58°C temperature was previously determined to be optimal for each of the primer pairs.

Figure 2-5. General scheme for quantitative RT-PCR (Tarnuzzer *et al.,* 1996). Template sequence competes against cell-derived mRNA for oligonucleotide primer binding. As visualized by ethidium bromide staining of agarose gels, the precise starting copies of mRNA can be determined.


RNA isolation
fetal / neonatal submandibular gland
GITC / CsCl ultracentrifugation
Aliquot
Add known copies of pMATRIX RNA to each tube
cDNA synthesis
PCR amplification
template sample RNA
Gel electrophoresis
Analyze densitometrically by ethidium bromide staining intensity on flatbed scanner

Separation of Products and Photographing of Gels

PCR products were separated on 1.5% agarose gels containing 25 ng/ml ethidium bromide at 100V for ~2 hours until the template and cellular RNA amplicons were clearly discernible. The gels were photographed for 0.5 seconds using a Polaroid MP-4 Land camera using Type 57 film.

Scanning Image and Final Analysis

Photographs were scanned and digitized on a Hewlett-Packard IIcx digital scanner and stored as TIFF files on a Macintosh computer. Band intensities were determined using NIH-Image (v1.54). These intensity values were then normalized for their molecular weight. The log of the ratio of band intensities within each lane was plotted against the log of the copy number of template added per reaction. Molecules of target messages were determined where the ratio of template and target band intensities was equal to 1. Use of NIH Image is outlined in Appendix C.

Statistical Analysis

All data was statistically analyzed by both Kruskall-Wallis one-sided ANOVA and Newman-Keuls Multiple Comparison Test using Number Cruncher Statistical System version 6.0 (NCSS; Hintze, 1995). For the K-W One-Way ANOVA, the null hypothesis was that all means were equal while the alternate hypothesis was that at least two of the means were different. Data was considered statistically significant at alpha = 0.05.

Immunohistochemistry

Immunohistochemical detection of each ECM protein was performed as previously described (Chegini *et al.*, 1993). A schematic diagram of the technique is shown in Figure 2-6. The goat anti-human/bovine collagens I, III, and IV polyclonal antibodies used at 1:20 were graciously supplied by Southern Biotechnology Associated (Birmingham, AL). The rabbit anti-mouse laminin B1/B2 used at 1:2000 was purchased from Upstate Biotechnolgy (Lake Placid, NY). The rabbit anti-mouse fibronectin used at 1:100 was purchased from Chemicon International (Temecula, CA). The rabbit anti-bovine lysyl oxidase used at 1:500 was generously supplied by Dr. Philip Trackman (Department of Periodontology and Oral Biology, Boston University). The slides were washed with phosphate-buffered saline and incubated for 1 hour at room temperature with either goat anti-rabbit or horse anti-goat biotinylated second antibody conjugate (Vector Laboratories, Burlingame, CA. The stained sections were photographed with an Olympus BH-2 microscope using Normarski interference contrast optics.

Figure 2-6. Schematic diagram of the immunohistochemical protocol. (adapted from Ausubel, 1987).

tissue specimen

fix overnight in 4% paraformaldehyde
dehydrate in ethanol series and xylene
embed in paraffin

cut tissue into 6µm thick sections
dry slides and store indefinitely

rehydrate section
inactivate endogenous peroxidase with 3% H_2O_2
block non-specific binding with normal serum

incubate 3 hrs with primary antibody

wash slide with PBS

incubate 1 hr with biotinylated secondary antibody

wash slide with PBS

incubate with streptavidin horseradish peroxidase conjugate
add diaminobenzidine and develop for 5-10 min

stop reaction in water

dehydrate section in a graded alcohol seried and xylene
cover sections with cover slip
photograph

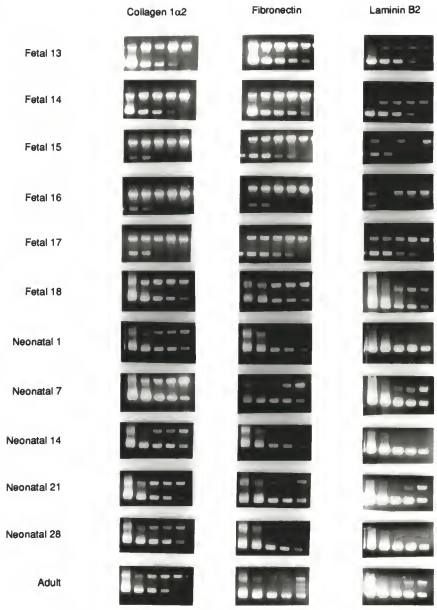
CHAPTER THREE
RESULTS

Synthesis of pMATRIX

A multiprimer template, pMATRIX, containing oligonucleotide sequences for collagens α2(I), α1(III), α1(IV), fibronectin, laminins B1 and B2, elastin, and lysyl oxidase has been synthesized according to the procedures described in the Materials and Methods. Using pMATRIX and competition-based quantitative RT-PCR it is possible to quantify the abundance of mRNA corresponding to any of the extracellular matrix genes in human, mouse, or rat tissues.

While constructing pMATRIX, to ensure that the multiprimer insert was complete and intact, all 3' and 5' primers were used to amplify products of the expected size. cRNA of the poly(A)-containing multiprimer insert was transcribed using Sp6 RNA polymerase. Final yields were 3.72×10^{11} copies RNA template/μl. Ten-fold serial dilutions were made in DEPC-treated water and standard RT-PCR reactions were performed using 5' and 3' oligonucleotide primers for fibronectin (MH61 and MH62). Products from these reactions, when separated on a 1.5% agarose gel, yielded decreasing amplified product corresponding to the decreased template copy number (data not shown). This simple approach demonstrated that PCR using decreasing template copy number resulted in a decrease in amplification products.

Figure 3-1. Representative composite data for Q-RT-PCR. Actual raw PCR data are shown for collagen α2(I), fibronectin, and laminin B2 for fetal (days 13-18), neonatal (days 1, 7, 14, 21, and 28), and adult submandibular glands. The product sizes are the same as listed in Table 2-1. Reactions in lanes 1-5 contain decreasing copies of pMATRIX template.


Collagen 1α2
Fibronectin
Laminin B2
Fetal 13
Fetal 14
Fetal 15
Fetal 16
Fetal 17
Fetal 18
Neonatal 1
Neonatal 7
Neonatal 14
Neonatal 21
Neonatal 28
Adult

The amount of total RNA recovered from fetal submandibular glands ranged from 1.2 μg RNA/gland to 4.4 μg RNA/gland for days 13 and 18, respectively. The major limitation of these experiments was the availability of fetal mouse salivary gland RNA. Yields of total RNA for neonatal (postnatal days 1, 7, 14, 21, and 28) and adult submandibular glands ranged from 50-300 μg/total RNA/gland and were not limited.

Quantitation of ECM Gene Expression Using pMATRIX

A composite of raw data for Q-RT-PCR from collagen α2(I), fibronectin, and laminin B2 from fetal day 13 to adult is shown in Figure 3-1. Quantitation is achieved by measuring the area of each PCR generated band, dividing the area by the molecular weight of each band, and plotting the log of the ratio of band intensities over the log of the copies of template. The resulting line should be linear with a slope of 1. A sample calculation for Figures 3-2 and 3-3 is detailed below. The intensities of the Q-RT-PCR products were measured for both the supertemplate and the cellular RNA in Figure 3-2. Lane 3 of the top panel of Figure 3-2 (Laminin B2) has a 677 bp band (RNA) with an area of 567 and a 344 bp band (supertemplate) with an area of 1582. Lane 4 has a 677 bp band with an area of 815 and a 344 bp band with an area of 245. Lane 5 has a 677 bp band with an area of 1506 and a 344 bp band with an area of 91. The band intensities were normalized by dividing the area by the MW such that 567/677 = 0.84, 1582/344 = 4.60, 815/677 = 1.20, 245/344 = 0.71, 1506/677 =2.22 and 91/344 = 0.26. The ratio of band intensities was then calculated (4.60/0.84 = 5.48, 0.71/1.20 = 0.59, and 0.26/2.22 =

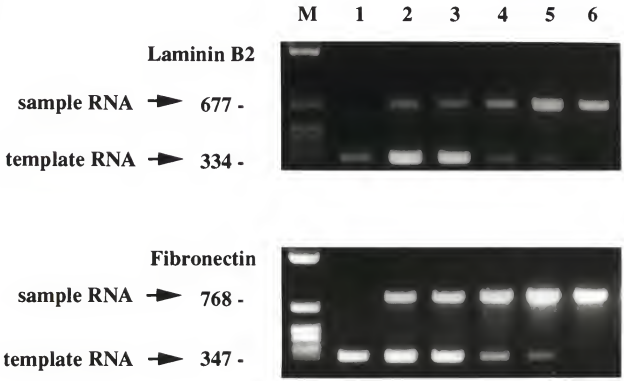


Figure 3-2. Ethidium bromide stained agarose gel showing data from fibronectin and laminin B2 Q-RT-PCR. The lanes (2-9) represent decreasing amounts of pMATRIX cRNA (6.42×10^5 to 3.3×10^2 copies, respectively) added to 1.5μg of total RNA. M marks the DNA marker lane (pGEM).

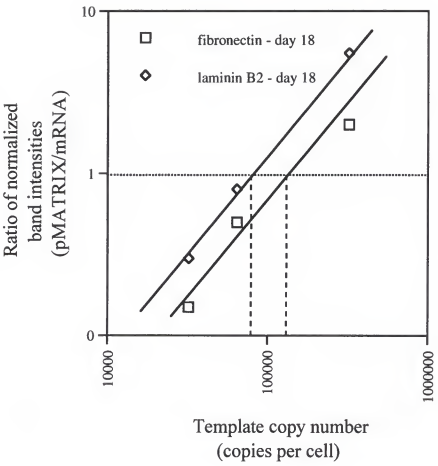


Figure 3-3. Graphed representation of the data from Figure 3-2 with the log of the ratio of the normalized band intensities plotted against the log of template copy number □ fibronectin - day 18; ◇ laminin B2 - day 18.

Figure 3-4. Copies of mRNA per cell for each ECM gene measured over the development of mouse submandibular glands.

copies mRNA molecules per cell
1000000
750000
500000
250000
0
Fetal 13
Fetal 14
Fetal 15
Fetal 16
Fetal 17
Fetal 18
Neonatal 1
Neonatal 7
Neonatal 14
Neonatal 21
Neonatal 28
Adult
Time (Day)
Collagen α2(I)
Collagen α1(III)
Collagen α1(IV)
Fibronectin
Laminin B2
Lysyl Oxidase
Elastin

0.12) and finally plotted as the log of these calculated ratios against the log of the starting copy number of the template added to each RT reaction. This should result in a line with a slope of 1. The number of RNA copies was determined from where the line crosses 1. With 26 pg of total RNA per mammalian cell (Bradhorst and McConkey, 1974), the value can then be expressed as copies of RNA per cell.

Using this method of quantitation, the ECM gene expression in developing mouse submandibular glands was measured. This analysis met all requirements previously stated in that the part of the calibration curve used for quantification should be linear and have a slope equal to 1. In practice, a correct standard curve can be considered a strong argument for equal rates of amplification (Raeymaekers, 1994; Raeymaekers, 1995; Siebert, 1993; Siebert and Larrick, 1992).

As shown in Figure 3-4, the number of collagen α2(I), collagen α1(III), and lysyl oxidase mRNA molecules per cell peaked on day 16 of development. For collagen a2(I), the number of copies of mRNA per cell at days 14, 15, 16, and 17 are significantly higher than other days and days 15 and 16 are significant over days 14 and 17 ($p < 0.05$). Collagen α1(III) mRNA expression was significantly higher at days 14 and 16 ($p < 0.05$). Collagen α1(IV) mRNA expression was below detectable limits except at day 13. The expression of elastin mRNA was only detectable on day 16 of development. Fibronectin and laminin B2 were more constitutively expressed but again had their highest copy number per cell at day 16. The levels of fibronectin mRNA was significant at day 16 above day 18, day 18 above days 15 and 17, and days 15 and 17 significantly higher than all other days ($p < 0.05$). Laminin mRNA was significantly higher at days 15

and 16 over days 14 and 17 with days 14 and 17 significantly higher than other days ($p < 0.05$). Lysyl oxidase mRNA was significantly higher at day 16 ($p < 0.05$). The null hypotheses that the means are equal can be rejected at $p < 0.0000001$ for collagens $\alpha 2$(I), $\alpha 1$(III), laminin B2, and lysyl oxidase and $p < 0.0000003$ for fibronectin. Although not statistically significant, a second smaller burst of ECM gene expression occurred for collagen $\alpha 2$(I), collagen $\alpha 1$(III), fibronectin, and laminin B2 at postnatal day 7, with the lowest overall ECM gene expression seen in the mature adult gland.

ECM Protein Localization by Immunohistochemistry in Developing Mouse Submandibular Glands

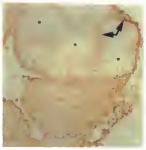
Immunhistochemistry was performed to localize each of these extracellular matrix proteins in fetal and adult submandibular glands (collagen type I, Figure 3-5A; collagen type III, Figure 3-5B; collagen type IV, Figure 3-5C; fibronectin, Figure 3-5D; laminin, Figure 3-5E; lysyl oxidase, Figure 3-5F. On day 16 of development the fetal tissue has immunoreactive collagen I, collagen III, and fibronectin present throughout the connective tissue between the epithelial lobules. Greater staining was seen in the outer mesenchymal capsule. There is little or no staining for these three proteins within the epithelial lobules. Immunoreactive lysyl oxidase and collagen IV were primarily in the mesenchymal capsule and basement membranes with some lighter staining present throughout the mesenchyme but like collagens I and III and fibronectin, were void in the epithelium. Immunoreactive laminin was present throughout both the mesenchyme, basement membranes, and the epithelium of the developing fetal submandibular glands.

Figure 3-5. Protein localization for ECM molecules.

a) Each ECM protein was localized in both day 16 developing glands and adult mouse submandibular by immunohistochemistry. Arrows indicate areas of more intense staining. Asterisks denote epithelial lobules.

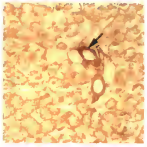
b) Controls for immunohistochemistry. The upper panel is a day 16 gland without primary antibody while the middle panel is an adult gland without primary antibody. The bottom panel is H&E staining of a day 16 developing mouse submandibular gland.

Collagen α2(I)

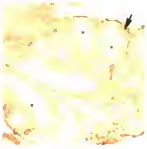


Fetal day 16

Adult

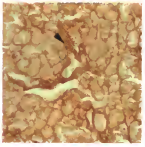


Collagen α1(III)

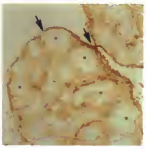


Fetal day 16

Adult

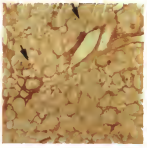


Collagen α1(IV)

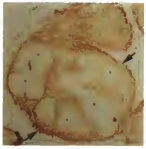


Fetal day 16

Adult

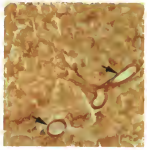


Fibronectin

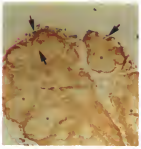


Fetal day 16

Adult



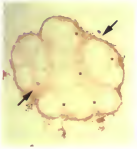
Laminin



Fetal day 16

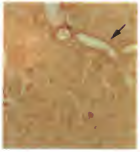
Adult

Lysyl Oxidase



Fetal day 16

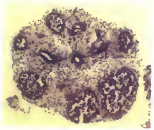
Adult



Controls

Day 16 -primary antibody

Adult -primary antibody



Day 16 H&E

Figure 3-5--continued.

Controls tissues which were treated with pre-immune sera were completely void of immunoreactive staining.

In the adult, immunoreactive collagen I and lysyl oxidase were present most strongly around the striated duct cells as well as the basal lamina surrounding the acini. There was no staining in the connective tissue septa. Collagen III also exhibited similar but stronger immunoreactive staining in the striated duct cells and in the basal lamina surrounding the acini than collagen I and lysyl oxidase. Immunoreactive fibronectin was most strongly associated with the striated ducts but was also present throughout the acini. Collagen IV was found most concentrated in the basal lamina of the acini and of the striated ducts. Laminin was most present in the striated ducts and basal lamina of the acini.

CHAPTER 4
DISCUSSION

Development of fetal submandibular glands involves the orchestrated expression of extracellular matrix molecules which direct the morphogenesis and cytodifferentiation of the epithelium. These events are highly regulated and coordinated both temporally and spatially (Cutler, 1989). Differentiation of salivary glands begins during fetal development when specific cells of the oral epithelium are induced to undergo organized, coordinated growth and to produce salivary-specific secretory proteins. Following this primary induction, the salivary gland rudiment initiates morphogenesis to develop its unique branching pattern. Once the primary branching patterns has been established, specific cells within the rudiment are induced to amplify the production of these salivary-specific secretory proteins. These primordial acinar cells modify their shape and their organelle composition to develop an architecture which is optimally suited to the synthesis and secretion of these exocrine products.

The full expression of morphogenesis and cytodifferentiation appears to be modulated or controlled by components of the extracellular matrix. The role of the collagens in submandibular gland development have been described but much less is known about the role of other extracellular matrix molecules. The use of competition-based quantitative RT-PCR to measure ECM gene expression provides valuable

information concerning the levels of extracellular matrix genes during the development of mouse submandibular glands.

A recent report measured the temporal levels of some ECM genes in the developing parotid gland of neonatal rats (Lazowski *et al.*, 1994). In that report standard Northern blot techniques were used which required 30 μg of total RNA and only relative levels of gene expression could be reported. It is an immense task to collect large quantities of total RNA from developing salivary glands. In comparison, by using competition-based quantitative RT-PCR to obtain quantitative information on each ECM mRNA levels, only 0.1 μg of total RNA per message was needed making it 100-300-fold more sensitive than traditionally used techniques. In addition the technique does not require radioactively-labeled probes and has the added convenience and speed of PCR. Competition-based RT-PCR permits an accurate calculation of the number of mRNA molecules in a sample and also allows for quantitative comparisons between different experiments.

The increases in ECM gene expression observed at both fetal day 16 and neonatal day 7 (Figure 3-4) occur immediately before two important functional changes in submandibular gland development. Beginning on fetal day 18, following the initial morphogenetic stage of salivary gland development, cytodifferentiation, or the initiation of specialized salivary-specific protein synthesis (*i.e.*, amylase and parotid-specific protein) within the branching structure, begins (Poulsen *et al.*, 1986). In addition the expression of muc-1 mucin, which correlates with epithelial differentiation during morphogenesis, is first seen at day 15 in developing mouse submandibular glands (Braga

et al., 1992). The dramatic increases in ECM gene expression at day 16 appear to represent the most dramatic changes in morphogenesis and the initiation of cytodifferentiation. Likewise the small peak in ECM gene expression observed at neonatal day 7 occurs just prior to the development of the stimulus/secretion coupling system that occurs at the suckling/weanling transition.

Recently, it was shown that glucocorticoids regulate morphogenesis in several branching organs including salivary glands. Jaskoll *et al.* (1994) studied the glucocorticoid receptor signal transduction pathway during mouse submandibular gland development and observed an increase in the number of branches in embryos after maternal injections of glucocorticoids. In addition the administration of glucocorticoids *in utero* enhanced the expression of acinar-cell-specific mucin protein. Another component of this study was the detection of transforming growth factor beta (TGF-β) 1, 2 and 3 mRNA transcripts in embryonic submandibular glands. Northern analysis suggested that the glucocorticoid signal transduction pathway modulates the rate of morphogenesis by regulating TGF-β 2 and 3 mRNA expression. This is an important observation since TGFβs are known to induce gene expression of extracellular matrix molecules including collagens, fibronectin, elastin, lysyl oxidase and TIMPS, as well as downregulate ECM-degrading enzymes (Macauley *et al.*, 1996c; Masságue, 1991). Glucocorticoids could then indirectly affect the branching morphogenesis by regulating the levels of TGF-βs, which in turn upregulate the expression and deposition of molecules making up the extracellular matrix.

Redman (1987) who noted that the high levels of laminin and collagen types α1(IV) and α1(I) expression at the early stages of neonatal parotid gland development may have indicated that the immature acinar and ductal cells and the mesenchyme produce these ECM components at a faster rate than of their mature counterparts in development. This observation has been validated by the quantitation of ECM gene expression in which fetal and neonatal submandibular gland gene expression was much higher than the adult. The changes in laminin and collagen expression suggests the involvement of these components in the profound changes in acinar and ductal cell morphogenesis, proliferation, and differentiation which take place during parotid gland postnatal development (Redman, 1987). The sharp decrease of laminin, collagens type α1(IV) and α1(I) seen at day 21 coincided with the onset of acinar and ductal cell cytodifferentiation. At this and later ages the basal surface of the cells have nearly contacted each other and the extracellular space has been diminished (Redman, 1987). Therefore one consequence of the decline of laminin and collagens I and IV may be the decrease of ECM due to displacement as the acini and the ductal cells attain proximity at the later stages of development (Lazowski *et al.*, 1994).

Although most basement membranes possess two scaffolding molecules, collagen type IV and laminin, several developing tissues produce basement membranes that initially lack type IV collagen but possess laminin (Cooper and MacQueen, 1983; Leivo *et al.*, 1980). This condition is observed both in the embryo and in capillary growth during tissue repair. Whereas the type IV collagen polymer becomes rapidly cross-linked on formation and is essentially irreversible, the laminin polymer can remain in a

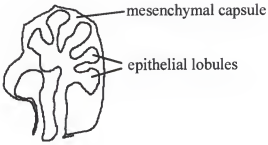
reversible state. Laminin, therefore, may be better suited as a matrix scaffolding in basement membranes that are undergoing rapid remodeling. It may be that laminin is the principle polymer of the developing mouse submandibular gland and explains why collagen α1(IV) levels were below detection even at day 16 when most ECM gene expression was optimal.

An increase in laminin expression might be expected in rapidly developing submandibular glands since the migration of fibroblasts has been shown to involve the interaction of the carbohydrate moieties of laminin with cell surface β1,4 galactosyltransferase (GalTase) (Begovac *et al.*, 1994). β1,4 GalTase is expressed in spatially-restricted, cell type-specific domains where it functions as a receptor for extracellular oligosaccharide ligands during migration and has been shown to be present on the leading and trailing edges of migrating cells. In addition it has been shown to bind to specific N-linked oligosaccharides within laminin. Increased expression of laminin as would be expected during highly proliferative stages of development such as the morphogenesis and cytodifferentiation of submandibular glands. Recently it was confirmed that cell-surface GalTase has the highest expression at day 16 fetal mouse submandibular gland development (Oxford *et al.*, 1995).

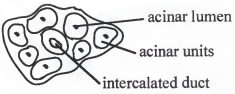
The immunohistochemical localization of the ECM proteins in mouse submandibular glands was consistent with earlier reports by Hardman and Spooner (1992), Spooner (1986), and Lazowski (1994). The distribution of the extracellular matrix proteins in both developing and mature salivary glands is shown in Figure 4-1.

Figure 4-1. Schematic representation of the expression of ECM proteins in developing mouse submandibular glands. Hatched areas represent the location of immunoreactive protein.
a) collagen I, collagen III, and collagen IV
b) fibronectin, laminin, and lysyl oxidase

Day 16

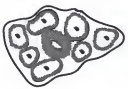


Adult

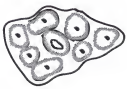


Collagen I

Collagen III



Collagen IV



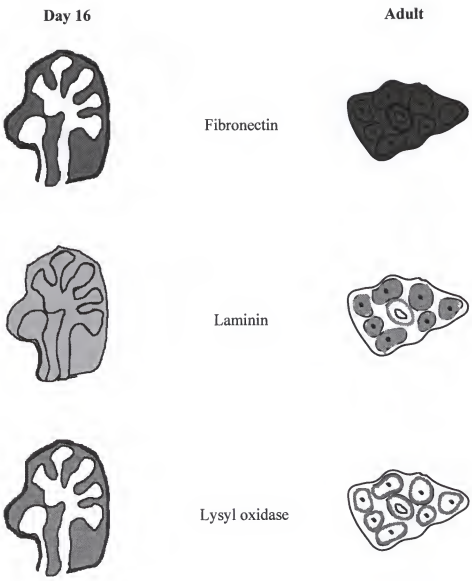


Figure 4-1--continued

Figure 4-2 reports on the localization of the ECM mRNAs. The work reported here was done to coincide and correlate with the data collected from the quantitative RT-PCR measurements of ECM gene expression. There was a difference noted however. In Hardman and Spooner (1992), laminin was found exclusively restricted to the basement membrane. Figure 3-5E however shows laminin, while primarily in the mesenchyme and basement membrane, also present at day 16 in the epithelium. In addition collagen IV was found, like Hardman and Spooner, to be present in basement membrane and the mesenchyme. The existence of basement membrane constituents such as collagen IV and laminin in the interstitial matrixes of developing tissues and embryos is not uncommon but is usually transient (Fitch and Linsenmayer, 1994). The first report of collagen IV not being found exclusively in the basement membrane in sheetlike arrangements was by Chen and Little who demonstrated that collagen IV was synthesized as "short wisplike" fibers by mouse embryonic lung mesenchymal cells and are not found within the basement membrane (Chen and Little, 1985, 1987). Kadoya and Yamashina demonstrated that the epithelium can transiently synthesize basement membrane specific molecules (laminin and collagen IV) before the formation of simple epithelial structures in the morphogenesis of mouse submandibular glands (1989). The transient presence of these molecules, which can be produced by either the epithelia or mesenchyme or both, seems to facilitate interactions between epithelial derived cells and the interstitial matrix during processes that involve tissue rearrangements, movement of individual cells, or anchorage of cells to the matrix.

Like the work of Nakanishi and Ishii (1989) and Nakanishi and colleagues (1986, 1988) collagen III appeared to be discontinuous along the epithelial-mesenchymal interface but it did not appear to be more prevalent at the clefts. The same polyclonal antibody (Southern Biotechnology Associates, Inc.) was used for this localization, however Nakanishi concentrated on earlier epithelial formation at days 12 and 13. With this information, Nakanishi proposed a working model on how the mesenchymal cells secrete collagen which exerts mechanical force to the epithelium giving strain to the surface of the epithelial lobule and initiating cleft formation. The temporal increases in collagen α1(III) do suggest that collagen III plays a major role in morphogenesis.

The literature reports that the extracellular matrix and in particular types I, III, and IV collagen, laminin, and chondroitin sulfate proteoglycan play roles in the control of glandular morphogenesis. With the exception of type IV collagen, and possibly laminin however, these molecules do not appear to be involved in the regulation of cytodifferentiation of salivary gland secretory cells. However, of the extracellular matrix molecules tested to date, only type IV collagen appears to play a significant role in the regulation of salivary gland secretory cell differentiation.

The expression of a number of extracellular matrix genes and their message localization in various developing tissues has been reported. (Andujar *et al.*, 1991; Lukinmaa *et al.*, 1993; McDonald and Tuan, 1989; Sarthy, 1993). In embryonic lung morphogenesis, laminin B1 and B2 was expressed evenly throughout the mesenchymal compartment and was higher in the epithelial buds (Thomas and Dziadek, 1994). Collagen αI(IV) was found throughout the epithelial layer and the mesenchyme. In that

report, like the results reported in Figure 3-5C, there is a concentration of staining on the basement membranes. The expression of fibronectin has been examined in rat fetal heart where again staining was evident in cells but also appeared to be localized to a thin layer along the basement membranes (Samuel *et al.*, 1994) . Laurie *et al.* (1989) examined the expression of laminin receptor, laminin, and collagen type IV in developing kidney. In that report it was found that the expression of laminin receptor mRNA preceded that of laminin and collagen type IV mRNAs. Again specific staining appeared to follow the basement membranes. Expression of laminin was examined in human fetal lung development by Lallemand *et al.* (1995) who showed that laminin is expressed by both epithelial and mesenchymal cells. Each of these reports is important in understanding the expected distribution of ECM mRNAs in developing tissues.

With the construction of the pMATRIX supertemplate and the advent of competitive Q-RT-PCR technology it has been possible to measure small changes in ECM gene expression during the development of fetal mouse submandibular glands. However, the ability to study gene expression from salivary gland tissues is not confined to development. With the coordinated expression of the extracellular matrix demonstrated using pMATRIX, other "thematic" plasmids (Figure 4-2) (Tarnuzzer *et al.*, 1996a), containing the sequences for growth factors, matrix metalloproteinases, cytokines, cell cycle proteins, and salivary-specific proteins, have been discussed. There are a number of other applications of these plasmids in dental sciences including studying periodontal disease, tumor biology, tooth morphogenesis, oral pathologies, and craniofacial development. Measuring gene expression from these minuscule tissues using

supertemplate plasmids and Q-RT-PCR could lead to a fundamental understanding of factors that contribute to both normal development and the initiation and progression of disease.

Figure 4-2. Potential list of other thematic supertemplates.

Epidermal Growth Factor Family
Transforming Growth Factor Beta

Epidermal growth factor
Transforming growth factor α
Transforming growth factor receptor
Transforming growth factor β1
Transforming growth factor β2
Transforming growth factor β3
Transforming growth factor βII-receptor

Platelet Derived Growth Factor Family
Fibroblast Growth Factors

Platelet derived growth factor A
Platelet derived growth factor B
Platelet derived growth factor receptor α
Platelet derived growth factor receptor β
acidic Fibroblast growth factor
basic Fibroblast growth factor
Fibroblast growth factor receptor

Matrix Metalloproteinases
Tissue-inhibitors of MMPs

Type I collagenase (MMP-1)
72 kD gelatinase (MMP-2)
Stromelysin (MMP-3)
PUMP-1 (MMP-8)
92 kD collagenase (MMP-9)
neutrophil elastase
Tissue-inhibitor of MMPs I (TIMP1)
Tissue-inhibitor of MMPs II (TIMP2)

Cell-cycle proteins

Cytokines

Interleukin 1α
Interleukin 1b
Interleukin 4
Interleukin 8
Interleukin 10
Tumor necrosis factor a
Tumor necrosis factor receptor I (p55)
Tumor necrosis factor receptor II (p75)
Interleukin 1 receptor antagonist
Interleukin 1 receptor (p68)

Salivary gland specific proteins

Histatin
Parotid-specific protein (PSP)
Amylase
Cystatin S
Proline-rich proteins
β1,4-Galactosyltransferase
Lysozyme
Lactoferrin
Mucin
Kallikrien

Extracellular Matrix

Collagen α2(I)
Collagen α1(III)
Collagen α1(IV)
Fibronectin
Laminin B1
Laminin B2
Elastin
Lysyl oxidase

Oncogenes

c-*erb*A
c-*erb*B
c-H-*ras*
c-*src*
c-*mos*
c-*raf*
c-*myc*
c-*fos*
c-*jun*

Signal-transduction factors

cAMP
Protein kinase A
Protein kinase C
MAP kinase
MAPK kinase
Protein lipase Cγ
Raf-1
GAP

Insulin-like Growth Factor Family

Insulin-like growth factor 1
Insulin-like growth factor 1 receptor
Insulin-like growth factor II
Insulin-like growth factor II receptor
Insulin-like growth factor binding protein 1
Insulin-like growth factor binding protein 2
Insulin-like growth factor binding protein 3
Insulin-like growth factor binding protein 4
Insulin-like growth factor binding protein 5
Insulin
Insulin receptor

CHAPTER FIVE
OTHER STUDIES

In addition to the research detailed in the preceding four chapters, numerous other collaborative research efforts have resulted in published manuscripts. The abstracts for each of these manuscripts is included here. As a March of Dimes Predoctoral Fellow in the Department of Biochemistry and Molecular Biology, the initial research centered on the cloning and characterization of the human lysyl oxidase gene. Later as a NEI trainee in the laboratory of Dr. Gregory Schultz, the research efforts shifted toward understanding the regulation of lysyl oxidase by growth factors in wound healing. In addition exciting work was done on characterizing growth factors in ocular and skin wound healing as well as in understanding the microenvironment of chronic wounds and how matrix metalloproteinases may play a role in the chronicity of these wounds. Finally as a NIDR Trainee in the Department of Oral Biology, the work shifted toward the dissertation project as well as identifying growth factors synthesized by the salivary glands and present in saliva. It is difficult to find a unifying theme for all of these research efforts but common to most of the recent manuscripts is the identification and molecular characterization of peptide growth factors present in wounds, tears, and saliva which may play an important role in the normal wound healing of skin, the eye, and the oral cavity, respectively.

My initial research project involved the cloning and characterization of the human lysyl oxidase gene (Svinarich *et al.,* 1992). This work was done exclusively in the laboratory of Thomas P. Yang with collaboration from Dr. Stephen Krawetz, Wayne State University. Lysyl oxidase is copper dependent, extracellular matrix enzyme which catalyzes the formation of aldehyde cross-links in elastin and collagen. Cross-link formation is initiated by the oxidative deamination of ε-amino groups of specific lysyl and hydroxylysyl residues. This forms reactive allysyl and hydroxyallysyl intermediates which undergo a series of spontaneous nonenzymatic condensation reactions to form novel covalent cross-links. These cross-linkages serve to fix the mechanical limits for tensile strength and elasticity of tissues. The inter- and intramolecular cross-linkages formed within the collagen and elastin matrix are essential for the maintenance of the structural integrity of most, if not all, tissues. Lysyl oxidase is thought to play a key role in restructuring the connective tissue matrix following episodic injury that occurs as part of atherosclerosis and pulmonary and hepatic fibrosis.

Another area of research involved the effects of TGF-β1 on the expression of three extracellular matrix genes, collagen α2(I), elastin, and lysyl oxidase in three different cultures of human fibroblasts (Macauley *et al.,* 1996). This work was completed in the laboratories of Dr. Thomas P. Yang, Department of Biochemistry and Molecular Biology, and Dr. Gregory S. Schultz, Department of Obstetrics and Gynecology. At the time of data collection, it was the first demonstration that lysyl oxidase, like other ECM genes, was regulated by TGF-β1. It also demonstrated that there was a concomitant

effect of TGF-β1 on collagen α2(I), elastin, and lysyl oxidase gene expression in the chronic state of laryngeal stenosis.

While in the Schultz laboratory a number of collaborative efforts were initiated that resulted in published manuscripts. The first project was in collaboration with Dr. Michael G. Humphreys-Beher in the Department of Oral Biology. This project sought to identify growth factors produced and secreted by the salivary glands and present in the saliva (Humphreys-Beher *et al.,* 1994). The presence of epidermal growth factor (EGF) has been known since it was isolated from mouse salivary glands in 1962. While the exact reason why the ductal cells of the salivary glands produce so much is not completely understood but it is proposed that EGF protects the oral and gut mucosa from the constant damage seen with mastication. The presence of other growth factors in the EGF family had not previously been reported. In this report, the levels of TGF-α were determined and compared with patients exhibiting oral pathologies, namely xerostomia and Padget's disease.

It has long been known that salivary constituents carry out a number of biological functions which are key to maintaining oral health. The proteins secreted by saliva aid in lubrication of the hard and soft tissues, digestion, remineralization, as well as provide antimicrobial activity. Moreover saliva contains factors that maintain mucosal integrity. The presence of EGF in saliva has been proposed to be an important factor in maintaining the integrity of oral and gut mucosa. EGF and nerve growth factor (NGF) have been recognized components of saliva (Cohen, 1962; Levi-Montalcini and Cohen, 1960), but

recently a number of manuscripts have reported the presence of a considerable number of other biologically active peptides in saliva. These include TGF-α (Humphreys-Beher *et al*., 1994a; Yeh *et al*., 1989), insulin (Kerr *et al*., 1995; Murakami *et al*., 1992; Smith and Patel, 1984), insulin-like growth factors I and II (IGF I and IGF II) (Costigan *et al*., 1988; Kerr *et al*., 1995; Ryan *et al*., 1992), transforming growth factor beta (TGF-β) (Amano *et al*., 1991; Jaskoll *et al*., 1994), and fibroblasts growth factor (FGF) (Hiramatsu *et al*., 1994). A review article which summarized this information was submitted (Zelles *et al.,* in press).

Like the protective functions growth factors provide the oral and gut mucosa, it is well established that tears provide substances that are critical for normal corneal physiology. In collaboration with Dr. Gysbert van Setten of the Karolinska Institute who determined that EGF was a component of tear fluid, it was determined that TGF-α was also synthesized by the lacrimal glands and was present in human tears (van Setten *et al.,* 1994, 1996). It is thought that with EGF, TGF-α is important in stimulating healing of epithelial injuries by promoting migration and mitosis of epithelial cells.

To further the understanding of the role of growth factors in ocular wound healing, a number of clinical trials have been performed. In addition to EGF and TGF-α, TGF-β1 has been examined for its role in ocular wound healing. In this manuscript, it was reported that when TGF-β1 was topically applied to corneal wounds, that there was an increase in tensile strength and a decrease in the rate of epithelial regeneration (Oxford *et al.,* in press). TGF-β1 also had positive effects on the levels of other growth factors

(EGF, TGF-α) as well as the EGF and type II TGF-β receptor present in the eye. The positive effects of TGF-β1 in corneal wound healing then are thought to occur in part through these changes in tensile strength and growth factor and receptor mRNA levels. In light of all the work done in the Schultz laboratory on the role of growth factors in ocular wound healing, a manuscript was completed which included all the known data linking numerous growth factors to roles in ocular wound healing (Schultz *et al.*, 1994).

Another aspect of research has been dealing with understanding the microenvironment of chronic wounds. Normal wound healing is characterized by a cascade of highly regulated, rate limiting steps. There are a number of possible regulatory steps in the fine balance between synthetic and degradative pathways in normal healing, that if altered, could result in chronic wound formation. Chronic wounds are clinically described as open wounds which do not heal by conventional methods. Factors which are known to contribute to the pathogenesis of chronic wounds include oxygen tension in the wound and levels of calcium. Another factor that is now understood to contribute to chronic wounds is the level of proteases, mainly the matrix metalloproteinases (MMPs). A normal part of wound healing is the degradation and turnover of the extracellular matrix and the synthesis of the new matrix in the form of a scar. In chronic wounds it was found that the levels of MMPs are very high resulting in the degradation of not only the ECM but other factors important in the wound healing process, *i.e.,* growth factors. One approach to bring back a normal balance of synthesis and degradation is to inhibit the MMPs using synthetic MMP inhibitors. Gelardin is a

dipeptide which fills the substrate binding site and inactivates MMPs. In this study numerous wound fluids collected from patients with open chronically non-healing wounds was compared to wound fluid from normally healing mastectomy incisions. The data has been compiled and is part of a series of manuscripts which will explore the differences found in the microenvironment of chronic wounds which may lead to their non-healing state (Macauley *et al.,* submitted; Tarnuzzer et al., submitted).

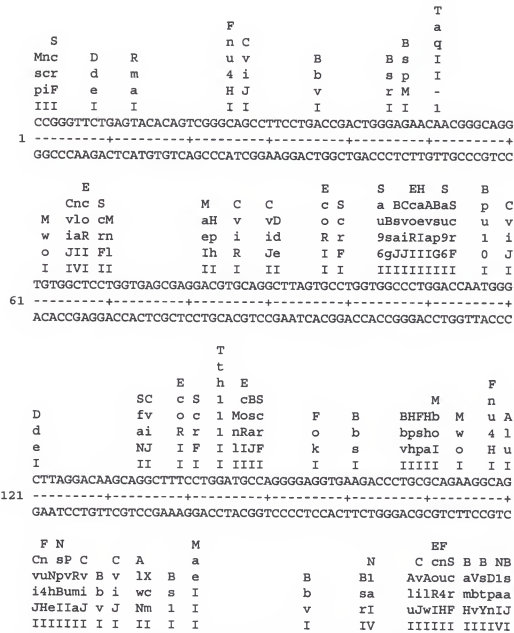
Finally, the technique of competition-based quantitative RT-PCR and the synthesis of the multisequence plasmids utilized for this technique are described in a manuscript. This report details the six plasmids which are have synthesized to date (Tarnuzzer *et al.,* 1996).

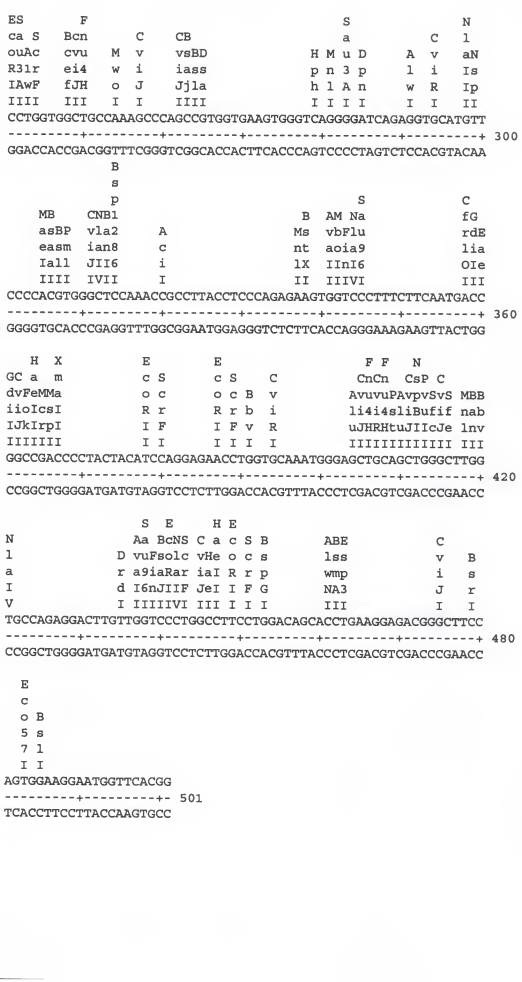
Together many of these manuscripts have demonstrated the importance of growth factors in the normal processes and healing of the skin, the eye, and the oral cavity.

APPENDIX A
LIST OF OLIGONUCLEOTIDES SYNTHESIZED FOR pMATRIX

gene	end	size (bp)	codes		
fibronectin	5'	21	MH61	MH137	MH179
	3'	21	MH62	MH138	MH180
	int	16	MH79		
elastin	5'	21	MH63	MH139	MH181
	3'	22	MH64	MH140	MH182
	int	16	MH80		
collagen α2(I)	5'	21	MH65	MH141	MH183
	3'	21	MH66	MH142	MH184
	int	16	MH81		
collagen α1(III)	5'	21	MH67	MH143	MH185
	3'	21	MH68	MH144	MH186
	int	16	MH82		
collagen α1(IV)	5'	21	MH69	MH145	MH187
	3'	21	MH70	MH146	MH188
	int	16	MH83		
GTAK	5'	21	MH71		
	3'	21	MH72		
	int	16	MH84		
laminin B1	5'	21	MH73		
	3'	21	MH74		
	int	16	MH85		
laminin B2	5'	21	MH75	MH147	MH189
	3'	21	MH76	MH148	MH190
	int	16	MH86		
lysyl oxidase	5'	21	MH77	MH149	MH191
	3'	21	MH78	MH150	MH192
	int	16	MH87		
plas 1		80	MH88		
plas 2		80	MH89		
plas 3		80	MH90		
plas 4		80	MH91		
plas 5		80	MH92		
plas 6		80	MH93		
plas 7		80	MH 94		
plas 8		81	MH95		
poly(A) tail 5'		30	MH104		
3'		30	MH105		

APPENDIX B
RESTRICTION MAP OF pMATRIX MULTIPRIMER INSERT




ES F S N
ca S Bcn C CB a C l
ouAc cvu M v vsBD H M u D A v aN
R31r ei4 w i iass p n 3 p l i Is
IAwF fJH o J Jjla h l A n w R Ip
IIII III I I IIII I I I I I I II
CCTGGTGGCTGCCAAAGCCCAGCCGTGGTGAAGTGGGTCAGGGGATCAGAGGTGCATGTT
241 ---------+---------+---------+---------+---------+---------+ 300
GGACCACCGACGGTTTCGGGTCGGCACCACTTCACCCAGTCCCCTAGTCTCCACGTACAA
B
s
p S C
MB CNB1 B AM Na fG
asBP vla2 A Ms vbFlu rdE
easm ian8 c nt aoia9 lia
Iall JII6 i lX IInI6 OIe
IIII IVII I II IIIVI III
CCCCACGTGGGCTCCAAACCGCCTTACCTCCCAGAGAAGTGGTCCCTTTCTTCAATGACC
301 ---------+---------+---------+---------+---------+---------+ 360
GGGGTGCACCCGAGGTTTGGCGGAATGGAGGGTCTCTTCACCAGGGAAAGAAGTTACTGG
H X E E F F N
GC a m c S c S C CnCn CsP C
dvFeMMa o c o c B v AvuvuPAvpvSvS MBB
iioIcsI R r R r b i li4i4sliBufif nab
IJkIrpI I F I F v R uJHRHtuJIIcJe lnv
IIIIIII I I I I I I IIIIIIIIIIII III
GGCCGACCCCTACTACATCCAGGAGAACCTGGTGCAAATGGGAGCTGCAGCTGGGCTTGG
361 ---------+---------+---------+---------+---------+---------+ 420
CCGGCTGGGGATGATGTAGGTCCTCTTGGACCACGTTTACCCTCGACGTCGACCCGAACC
S E H E
N Aa BcNS C a c S B ABE C
l D vuFsolc vHe o c s lss v B
a r a9iaRar iaI R r p wmp i s
I d I6nJIIF JeI I F G NA3 J r
V I IIIIIVI III I I I III I I
TGCCAGAGGACTTGTTGGTCCCTGGCCTTCCTGGACAGCACCTGAAGGAGACGGGCTTCC
421 ---------+---------+---------+---------+---------+---------+ 480
CCGGCTGGGGATGATGTAGGTCCTCTTGGACCACGTTTACCCTCGACGTCGACCCGAACC
E
c
o B
5 s
7 l
I I
AGTGGAAGGAATGGTTCACGG
481 ---------+---------+- 501
TCACCTTCCTTACCAAGTGCC

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BIOGRAPHICAL SKETCH

Shawn Patrick Macauley was born on August 20, 1964, in Malone, New York, and is the son of Karen and Curtis Winters of North Bangor, New York, and the late Edward Miles Macauley. He is the oldest of four siblings including one brother, Shannon Timothy, and two sisters, Kelli Erin and Courtney Anne. On June 18, 1988, Shawn married Beth Lynn Martin at First United Methodist Church in Port Saint Lucie, Florida. Their children Erin Elizabeth (December 11, 1990) and Emily Logan (February 23, 1995) were both born in Gainesville.

In 1982, Shawn graduated with high honors from Franklin Academy Senior High School, Malone, New York, after which he entered the University of Florida and received his Associate of Arts and Bachelor of Science in Agriculture degrees from the Department of Microbiology and Cell Science in May 1987. He continued his studies in the Department of Microbiology and Cell Science in the laboratory of Dr. James F. Preston, III, where he studied the substrate specificities and phylogenetic relatedness of a group of alginate-degrading marine bacteria. He received his Master of Science degree in December 1989.

In January 1990, Shawn entered the Ph.D. program in the University of Florida College of Medicine and was awarded a prestigious March of Dimes Predoctoral Fellowship from 1990-1992 while working in the laboratories of Dr. Thomas P. Yang, Department of Biochemistry and Molecular Biology, and Dr. Gregory S. Schultz, Institute for Wound Research. In August 1993, Shawn transferred into the laboratory of Dr. Michael G. Humphreys-Beher, Department of Oral Biology. In 1995 he was a winner of an AADR Edward H. Hatton Award and later won 2nd place in the IADR Edward H. Hatton Award Competition in Singapore.

During his University of Florida career, Shawn was active in a number of instrumental and choral groups including the University of Florida Marching Band, Concert Band, and Men's Glee Club. Shawn was also a member of the 1985-86 Up With People Cast D during which time he was enrolled at the University of Arizona, Tucson. As a soloist and saxophonist he represented the United States in the cast's year-long tour across the U.S., including the Superbowl XX halftime show in New Orleans, Ireland, Northern Ireland, Belgium, Luxembourg, Holland, France, and Japan. In 1988 he was inducted into the University of Florida Circle of Omicron Delta Kappa Honor Society.

At University United Methodist Church and Student Center, Shawn was a collegiate staff member from 1983-1988 and a member of the Maranatha, Loft, and Omega Handbell Choirs from 1982-1993. In 1991 he traveled to San Cristobal, Dominican Republic, to help build and repair two orphanages. More recently he has been

singing tenor with the Gainesville Civic Chorus and playing saxophone in the Worship Ensemble at Creekside Community Church and with Salsa Byte, a local Latin band.

Following graduation, Shawn has accepted a faculty position in the Department of Genetics and Cell Biology at Washington State University where he will establish his own research program at the Health Research and Education Center as well as coordinate new graduate programs in genetic counseling and microbiology to begin in the 1998 or 1999 school years. His wife Beth is completing her Ph.D. in Communication Processes and Disorders with a minor in neuropsychology and will be an Assistant Professor of Speech and Hearing Sciences at Washington State University.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Associate Professor of Oral Biology

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Linda L. Brinkley
Professor of Oral Biology

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Professor of Oral Biology

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements of the degree of Doctor of Philosophy.

May, 1996

Dean, College of Medicine

Dean, Graduate School